PHENOTYPIC AND GENETIC DIVERSITY OF PSEUDOMONADS ASSOCIATED WITH THE ROOTS OF FIELD-GROWN CANOLA

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By

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ABSTRACT

Pseudomonads, particularly the fluorescent pseudomonads, are common rhizosphere bacteria accounting for a significant portion of the culturable rhizosphere bacteria. The presence and diversity of *Pseudomonas spp*. in the rhizosphere is important because of their ability to influence plant and soil health. Diversity is generated as the result of mutation, the rearrangement of genes within the genome and the acquisition of genes by horizontal transfer systems, e.g. plasmids, bacteriophages, transposons or integrons. The purpose of this study was to examine the phenotypic and genotypic diversity of a subset of pseudomonads (N=133) isolated from the rhizosphere and root-interior of four cultivars of field-grown canola. Pseudomonads were analyzed according to their 16S rRNA and *cpn*60 gene sequences and selected phenotypic properties (fatty acid methyl ester (FAME) profiles, antibiotic resistance, extracellular enzyme production and carbon substrate utilization). On the basis of 16S rRNA and *cpn*60 gene sequences, two major clusters were observed, the *Pseudomonas fluorescens* complex and the *P. putida* complex. Phylogenetic analysis of the partial gene sequences suggested that the phylogeny of root-associated pseudomonads had no effect on their associations with different cultivars or root zones (i.e. rhizosphere and root interior). Principal component analysis (PCA) of their phenotypic properties revealed little variation among the pseudomonads associated with different canola cultivars. Importantly, while little difference was observed in isolates from different cultivars significant phenotypic

variation was observed in isolates from different root zones. Cluster analysis of their phenotypic properties exhibited little correlation with their phylogenetic relationships. In the majority of situations, the isolates grouped into a phylogenetic cluster had less than 75-80% similarity among their phenotypic traits despite a close evolutionary relationship as determined by 16S rRNA and *cpn*60 gene sequencing. The results indicated that the genotype of the rhizosphere pseudomonads was not accurately reflected in their phenotype. Analysis of the mobile genetic elements (MGEs) associated with a randomly selected subset of the pseudomonad isolates (N=66) revealed that 58% (N=38) contained plasmids, 50% (N=33) contained inducible prophages, 24% (N=16) contained integrons and 23% (N=15) contained transposons. Examination of the MGEs associated with a subset of rhizosphere pseudomonads revealed that MGEs were present in the isolates independent of the degree of similarity between their phenotypic and phylogenetic relationships. Therefore, mutation and genomic rearrangement appear to be the major influences in the observed incongruence between the phylogenetic and the phenotypic relationships of the bacteria examined.

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LIST OF ABBREVIATIONS

-	Negative
+	Positive
%	Percentage
µg	Microgram
µL	Microlitre
µM	Micromolar
ACC	1-Aminocyclopropane-1-carboxylic acid
ANOVA	Analysis of variance
Amp	Ampicillin
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
Cb	Carbenicillin
Cm	Chloramphenicol
CFU	Colony forming units
CS	Conserved segment
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAME	Fatty acid methyl ester
g	Gram
Gm	Gentamycin
h	Hours
Hg	Mercuric chloride

IAA	Indole-3-acetic acid
IR	Inverted repeats
Km	Kanamycin
kb	Kilobases
LB	Luria-Bertani
M	Molar
Mbp	Megabase pairs
MGEs	Mobile genetic elements
mg	Milligram
MIDI	Microbial ID, Inc.
mL	Millilitre
mM	Millimolar
MVSP	Multivariate statistics package
Na	Nalidixic acid
NK	Not known
OD	Optical density
OTU	Operational taxonomic units
P1	Promoter 1
P2	Promoter 2
PCA	Principal component analysis
PCR	Polymerase chain reaction
PGPR	Plant growth-promoting rhizobacteria
PHYLLIP	Phylogeny inference program

Pm	Phenylmercury acetate
Psv	Pairwise similarity values
R	Resolvase
rDNA	Ribosomal deoxyribonucleic acid
RDP	Ribosomal database project
SDS	Sodium dodecyl sulfate
rcf	Relative centrifugal force
Res	Recombination site
RNA	Ribonucleic acid
sec	Seconds
SIM	Similarity indices
Sm	Streptomycin
spp.	Plural species
sp.	Species
Su	Sulphonamides
TBE	Tris-boric acid-EDTA
Tc	Tetracycline
Tn	Transposon
UV	Ultra-violet

1.0 INTRODUCTION

Bacteria are an exceptionally diverse group of organisms exhibiting high levels of variability among genome content and structure, metabolic properties, cellular structure and lifestyles even within species (Ochman et al., 2000). The genus *Pseudomonas* within the γ-Proteobacteria has been suggested to be the most diverse and ecologically significant group of culturable bacteria (Spiers et al., 2000). *Pseudomonas spp*., collectively referred to as pseudomonads, constitute a large group of highly diverse bacteria found in large numbers in all natural environments (terrestrial, freshwater and marine) and some form associations with plants and animals. Their ability to colonize a wide range of habitats suggests a remarkable degree of physiological and genetic adaptability.

The extent of their physiological diversity was initially realized by Stanier et al. (1966) who found that pseudomonads have the capacity to degrade a wide range of substrates including aromatic compounds, halogenated derivatives and recalcitrant organic residues. Since then several reports have expanded our knowledge in regard to the physiological diversity of pseudomonads (Clarke, 1982; Timmis & Pieper, 1999; Martins dos Santos et al., 2004). For example, Madigan et al. (1997) reported that some *Pseudomonas* species utilize more than 100 different compounds for sources of carbon and energy while others are only able to use 20.

The genetic diversity found within pseudomonads gives rise to a wide range of phenotypes. Increasing evidence suggests that the diversity of genome architecture (chromosomes and accessory genetic elements) is of particular importance (Jablonka et al., 1998; Kirschner & Gerhart, 1998; Gogarten et al., 2002). Examination of the deoxyribonucleic acid (DNA) fingerprints of *Pseudomonas spp*. reveals a high level of polymorphism among strains of a species and among strains highly related on a phenotypic basis (Ginard et al., 1997; Rainey et al., 1994). For example, substantial genome size polymorphism exists between multiple isolates from a single species; *P. stutzeri* spans from 3.7 to 4.7 Mbp (Ginard et al., 1997) and *P. aeruginosa* spans from 5.2 to 7.1 Mbp (Schmidt et al. 1996).

Several papers document microbial diversity at the community and at the organism level, but few examine the causes or ecological significance of this diversity. Diversity arises and is maintained through the interaction between ecological and genetic factors. The ultimate cause of diversification is mutation, but the variation generated by both mutation and recombination (including lateral gene transfer) is sorted and shaped by selective pressure and genetic drift (Spiers et al., 2000). Genetic factors are the ultimate determinants of patterns of diversity for without genetic variation there can be no evolution or divergence. Mutation and selection alone are powerful evolutionary forces, but nevertheless the ubiquitous nature of *Pseudomonas spp*. leads many to wonder if they are inherently more evolvable or if lateral gene transfer is largely the cause of their large diversity.

Misko and Germida (2002) examined the taxonomic and functional diversity of pseudomonads associated with the roots of field-grown canola. They found

pseudomonads accounted for about 35% of the culturable root-associated bacteria. Characterization of carbon substrate utilization profiles indicated that these pseudomonads were metabolically diverse and isolates shown to be the same strain by fatty acid methyl ester (FAME) analysis exhibited significant metabolic diversity, suggesting functional redundancy among these pseudomonad isolates. In light of their results, my Ph.D. thesis research initiated a project to examine the variation exhibited between the physiological and the genetic diversity of these pseudomonad isolates. The main objectives of my study were to compare the physiological properties and the phylogenetic relationships of these pseudomonad isolates and to examine the diversity of their associated mobile genetic elements (MGEs). I hypothesized that the presence of MGEs within pseudomonad isolates would correlate with discrepancies observed between their phylogenetic and physiological relationships.

Before examining the mobile genetic elements associated with the pseudomonad isolates, it was necessary to confirm the findings of Misko and Germida (2002). As described in Section 3, I used 16S rRNA and *cpn*60 gene sequencing to confirm the taxonomic identification and diversity of the pseudomonads isolated from the roots of field-grown canola. In section 4, the pseudomonads were analyzed according to their 16S rRNA and *cpn*60 gene sequences and selected phenotypic properties (antibiotic resistance, extracellular enzyme production and carbon substrate utilization). I postulated that MGEs would be present in pseudomonads that differed in their phylogenetic and phenotypic relationships in comparison to the other pseudomonads. In addition, I postulated that MGEs could be responsible for the localization of pseudomonads to

specific root zones. In Section 5, I examined the MGEs associated with the root-associated pseudomonads.

My thesis is presented as three complete papers (Sections 3 through 5). Each section consists of an Introduction, Materials and Methods, Results and a Discussion. Due to the nature of this study the Materials and Methods are often used in all three studies; when this occurs readers will be referred back to the section that includes the original description. Any deviations from the original methodology will be noted. Section 6 is a summary of the key results from each section and some general conclusions.

2.0 LITERATURE REVIEW

2.1 Introduction – The rhizosphere

Plant life is the primary energy source that drives the terrestrial soil ecosystem. Due to the paucity of biologically available (i.e. the degree to which a contaminant or nutrient in can be absorbed, transported, and utilized physiologically) nutrients in the soil, plant roots must explore large volumes of soil to obtain the nutrients they require for growth and survival. As a result, plant roots provide a supply of biologically available nutrients to a large range of microorganisms in their immediate vicinity (rhizosphere effect). The plant roots can be divided into three zones of influence: the rhizosphere, the volume of soil surrounding the root that is subject to influence by the root (Hiltner, 1904), the rhizoplane, the surface of the root and any strongly adhering soil particles, and the root interior. Bacteria capable of colonizing the root interior are collectively referred to as endophytes.

Plant roots excrete various organic substances into the rhizosphere such as sugars, amino acids, vitamins etc. that are collectively referred to as exudates. The composition of the exudates varies with plant species, physiological condition of the plant such as age and nutritional status, and abiotic conditions such as temperature, water content, soil structure and aeration (Campbell and Greaves, 1990). Root exudates within the rhizosphere support the growth and metabolism of diverse microbial populations inhabiting this soil zone. The importance of the rhizosphere arises from the release of

exudates from the root and the resulting influence of increased microbial activity on nutrient cycling and plant growth (beneficial, neutral, harmful or variable).

2.1.1 Rhizosphere microbial biodiversity

Biological diversity, or biodiversity, is defined as the set of species, their genetic material and the ecosystem they belong to and it includes examination at the ecosystem, species and genetic diversity levels (Ohtonen et al., 1997). The impact of microbial diversity on the stability of ecosystem functioning has been studied intensely with relationships often observed between microbial diversity, soil and plant quality and ecosystem sustainability (Abawi and Thurston, 1992; Doran et al., 1996; Garbeva et al., 2004a). Rhizosphere microbial communities are often difficult to fully characterize, mainly because of their immense phenotypic and genotypic diversity, heterogeneity, and crypticity. Bacteria are the most numerous inhabitants of the rhizosphere, with population numbers typically ranging between 10^6-10^9 g^{-1} of rhizosphere soil. The number of bacteria associated with plant roots (per gram of soil) is 10- to 100- fold greater than the bacterial density associated with bulk soil (Lynch, 1990). On the basis of traditional cultivation studies, prokaryotic organisms typically found in soil and the rhizosphere are the *Proteobacteria* (*Pseudomonas*, *Serratia*, *Enterobacter* and *Rhizobium spp*.), the Gram-positive bacteria (*Clostridium spp*., *Bacillus spp*., *Arthrobacter spp*., *Brevibacterium spp*., and *Corynebacterium spp*.) and the Cytophaga/Flavobacteria/*Bacteroides* (CFB) group. Analysis of soils by culture-independent techniques has shown that *Proteobacteria* dominate 16S rDNA clone libraries using template DNA from a Queensland soil (Liesack & Stackebrandt, 1992; Stackebrandt et al. 1993). *Pseudomonas spp*. are the most studied of the rhizobacteria due to their ease of cultivation and the fact that they account for a significant portion of

the culturable rhizosphere population (Pankhurst, 1997; Garbeva et al., 2004b). Furthermore, Misko and Germida (2002) and Siciliano and Germida (1999) found that members of the genus *Pseudomonas* were the most commonly occurring bacteria in the rhizosphere of canola plants. The ability of *Pseudomonas spp.* to dominate the rhizobacterial population is typical of many plant species, such as sugar beet (Rainey et al., 1994), flax (Lemanceau et al., 1995) and tomato (Lemanceau et al., 1995), and suggests that they are the most diverse and ecologically significant group of culturable rhizobacteria.

2.2 *Pseudomonas*

Pseudomonas spp. are especially competitive in the rhizosphere and comprise a large portion of the total culturable bacterial population on the root. The majority of root-associated pseudomonads are saprophytic, and some species can interact with roots as either mutualists or pathogens. As rhizobacteria, they contribute to the cycling of nutrients, to the remediation of soils contaminated with organic pollutants, to plant growth promoting behaviour and by acting as biological control agents inhibiting several phytopathogens.

Some *Pseudomonas* species contribute to the cycling of carbon by degrading low molecular weight organic compounds, whereas others metabolize more complex molecules such as various aromatic compounds (Campbell et al., 1995b; Kersters et al., 1996). Stanier et al. (1966) documented the ability of some *Pseudomonas spp*. to utilize over 146 different organic compounds as a sole source of carbon and energy.

Pseudomonads also contribute to the cycling of nitrogen in the rhizosphere. Bolton et al. (1993) reported the production of the extracellular enzymes, urease and nitrate- and nitrite-reductases, which are responsible for the mineralization of organic

nitrogen to plant- and bacterial-available forms and the reduction of nitrate through nitrite to nitrous oxide, respectively. Rhizosphere and soil pseudomonads are also involved in the assimilation of nitrate or in the reduction of nitrate under oxygen limiting conditions (Clays-Josserand et al., 1999; Clays-Josserand et al., 1995; Carter et al., 1995). The majority of pseudomonads are considered to be aerobic, but some pseudomonads are facultative anaerobes as they reduce oxidized inorganic nitrogen compounds (i.e. NO_3^-, NO_2^-) by using them as terminal electron acceptors. This process is known as denitrification, and bacteria capable of the metabolic process are referred to as denitrifiers. Recently, Cheneby et al. (2000) found that of the culturable denitrying bacteria, *Pseudomonas spp*. were commonly isolated from agricultural soils.

The metabolic versatility of pseudomonads makes them attractive candidates for bioremediation of sites contaminated with organic pollutants. Many xenobiotic catabolic pathways, such as naphthalene, 2,4 - dichlorophenoxyacetic acid and atrazine degradation are plasmid-encoded (DiGiovanni et al., 1996; De Souza et al., 1998; Hohnstock et al., 2000). These pathways are of ecological and environmental importance because of the large number of xenobiotics introduced into the environment. Some of the introduced xenobiotics are readily degraded by microbial populations, whereas others are recalcitrant. Several catabolic plasmids have been isolated from bacteria, the principal source being bacteria isolated from soil (Sayler et al., 1990). The majority of these plasmid-bearing strains are Gram-negative *Pseudomonas spp*. (Sayler et al., 1990).

Pseudomonas spp. able to indirectly or directly promote plant growth are referred to as plant growth promoting rhizobacteria or PGPR (Kloepper et al., 1989). Indirect growth promotion occurs when the PGPR prevent or lessen the effects of pathogenic or deleterious microorganisms. These indirect effects occur as a result of antibiosis, induced

systemic resistance, or competition. Direct plant growth promotion entails either the production of a plant beneficial compound by the bacterium or the facilitation of nutrient uptake from the environment. Mechanisms of direct plant growth promotion include phytohormone production, nitrogen fixation, and the facilitation of nutrient uptake by siderophore production and by the secretion of organic acids that solubilize nutrients for uptake. Several species of pseudomonads promote plant growth (Glick, 1995; Shishido and Chanway, 2000). Antibiotic-producing pseudomonads have been implicated in the biological control of take-all disease of wheat (Thomashow and Weller, 1988; Brisbane et al., 1989). Shah et al. (1998) found that plant growth promoting *Pseudomonas* strains contain the genes for ACC (1-aminocyclopropane-1-carboxylic acid) deaminase. These genes catabolize ACC into ammonia and α-ketobutyrate prior to its conversion into ethylene. High levels of ethylene inhibit plant development whereas low levels of ethylene result in the promotion of plant growth and subsequent root elongation.

Due to the ubiquity and versatility of pseudomonads, there is considerable interest in exploiting these bacteria for a diverse range of biotechnological applications, such as the improvement of fossil fuels by desulphurization (Galan et al., 2000), the remediation of contaminated sites (Dejonghe et al., 2000) and as agents of plant growth promotion and plant pest control (Walsh et al., 2000). Despite the extensive examination of *Pseudomonas*, there is a lack of knowledge concerning the degree and source of *Pseudomonas spp*. diversity.

2.2.1 General characteristics

The family *Pseudomonadaceae* includes Gram-negative, non-spore forming, straight or slightly curved rods belonging to the genus *Pseudomonas*. They are typically motile by means of one or more polar flagella. Their oxidase reaction is usually positive

and their mol% G + C range is between 58-70. Generally, common to all constituent species of the genus *Pseudomonas* are certain physiological properties such as chemoorganotrophic nutrition, inability to grow under acidic conditions, aerobic metabolism, absence of fermentation, absence of photosynthesis, inability to fix nitrogen and capacity for growth at the expense of a large variety of organic substrates. There are a few exceptions to these standardized criteria of definition or identification. For example, *Pseudomonas stutzeri* is able to fix nitrogen and *Pseudomonas aeruginosa* is capable of anaerobic respiration utilizing NO_3^- as a final electron acceptor (denitrification) (Sylvia et al., 1998). Growth of these organisms occurs at neutral pH, under mesophilic conditions (Madigan et al., 1997). In addition, *Pseudomonas spp*. generally harbour multiple mobile genetic elements (MGEs) such as conjugative or transmissible plasmids, transposons and prophages (Holloway, 1969; Sayler at al., 1990; Campbell et al., 1995a, 1995b; Tan, 1999).

2.2.2 Classification and taxonomy

Bacterial classifications were devised for the microbiologists and not for the entities being classified (Staley and Krieg, 1984). Therefore, the concept of species must be regarded as an artificial concept that attempts to create units of biological diversity. The 'species problem' arises because no universal set of parameters has been recognized that most accurately classifies bacteria into species (Rosselló-Mora, 2003). The species concept for prokaryotes has evolved in parallel to the development of more sophisticated methodologies by which to retrieve useful taxonomic information with phenetic classifications dominating early taxonomic classification. With the advent of molecular methods, especially DNA hybridization and sequencing, classifications based on the inference of phylogenetic relationships have become commonplace (Palleroni, 2003).

Currently in the literature, there are at least three different species definitions: (i) taxospecies, defined as a group of organisms with mutually high phenotypic similarity that form an independent phenotypic cluster, (ii) genomic species, a group showing high DNA-DNA similarity and (iii) nomenspecies, as a group that bears a binomial name (Colwell et al., 1995). The taxospecies, based on phenetic classification, aims to create clustered groups of strains and genera based on overall phenotypic similarities and can be considered to indicate evolutionary relationships in the sense of showing the end products of evolution (Cowan, 1978). Given this, it would be expected that the phenetic criteria used for bacterial classification would be largely conserved among organisms related by evolutionary descent. This situation is not universal as genetic gain, loss, modification or gene transfer can lead to incongruence between phylogenetic and phenotypic traits that are important for taxonomic classification (Forney et al., 2004). To incorporate both approaches into current taxonomic classification a phylo-phenetic species concept has been suggested as a 'monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristics and is diagnosable by a discriminative phenotypic property' (Roselló-Mora and Amann, 2001).

The genus *Pseudomonas*, which was described in 1894 by Migula as a genus of Gram-negative polarly flagellated strictly aerobic rod-shaped microorganisms, has been subject to repeated taxonomic revisions (Palleroni, 1992; 1993). Defined in this way, the genus was very heterogeneous with a large number of species. In 1984, there were over 100 species included within the genus as listed in *Bergey's Manual of Systematic Bacteriology* (Palleroni, 1984). In March 2006, 170 species and subspecies of the genus *Pseudomonas* were validated on the Approved Lists of Bacterial Names

(www.dsmz.de/bactnom/bactname.htm). The taxonomy of the genus *Pseudomonas* has changed dramatically in recent years during the transition between classification based on phenotypic properties (e.g. *Bergey's Manual of Systematic Bacteriology*,1st ed., 1986) and classification based on genotypic (phylogenetic) properties (e.g. *Bergey's Manual of Systematic Bacteriology*, 2nd ed., 2001). The inclusion of DNA-RNA hybridization and DNA sequencing into *Pseudomonas* taxonomy has generated a phylo-phenetic concept that has improved the classification of pseudomonads into species. The utilization of DNA-RNA hybridization techniques revealed five rRNA homology groups among *Pseudomonas spp*. (Palleroni et al., 1973; Krieg and Holt, 1984; Palleroni, 1984). Organisms in rRNA homology groups II to IV originally classified as species of the genus *Pseudomonas* solely by phenetic means have been reclassified to the genera *Acidovorax*, *Aminobacter*, *Brevundimonas*, *Burkholderia*, *Commamonas*, *Halomonas*, *Herbaspirillum*, *Hydrogenophaga*, *Marinobacter*, *Methylobacterium*, *Oligotropha*, *Pseudoalteromonas*, *Ralstonia*, *Sphingomonas*, *Stenotrophomonas*, *Telluria*, *Vogesella* and *Zavarzinia* (Anzai et al., 2000). These analyses and taxonomic rearrangements identified rRNA homology group I species, including the type species *P. aeruginosa* and other species such as *P. fluorescens*, *P. putida*, and *P. syringae*, as members of a phylogenetically homogeneous group referred to as *Pseudomonas sensu stricto* (Palleroni, 1992; Moore et al., 1996). This classification is in agreement with phylogenetic information obtained from 16S rRNA, *gyr*B and *rpo*D sequencing (Anzai et al., 2000; Yamamoto et al., 2000) (Table 2.1).

2.3 Phenotypic classification and characterization of bacteria

Table 2.1. Current taxonomic classification or phylogenetic affiliation of members of the genus *Pseudomonas* as determined by their 16S rRNA gene (adapted from Anzai et al., 2000).

Proteobacteria subclass[a]	Previous name[a]	Current classification or phylogenetic affiliation	Reference	Accession No.
α	*Pseudomonas abikonensis*	*Sphingomonas* rRNA lineage	Anzai et al. (2000)	AB021416
	Pseudomonas aminovorans	*Aminobacter aminovorans*	Kersters et al. (1996)	
	Pseudomonas carboxydohydrogena	*Bradyrhizobium* group rRNA lineage	Anzai et al. (2000)	AB021393
	Pseudomonas carboxydovorans	*Oligotropha carboxidovorans*	Kersters et al. (1996)	
	Pseudomonas compransoris	*Zavarzinia compransoris*	Kersters et al. (1996)	
	Pseudomonas diminuta	*Brevundimonas diminuta*	Kersters et al. (1996)	AB021415
	Pseudomonas echinoides	*Sphingomonas* rRNA lineage	Kersters et al. (1996)	AB021370
	Pseudomonas extorquens and *Pseudomonas rosea*'	*Methylobacterium extorquens*	Kersters et al. (1996)	D32224
	Pseudomonas mesophilica	*Methylobacterium mesophilicum*	Kersters et al. (1996)	D32225
	Pseudomonas paucimobilis	*Sphingomonas paucimobilis*	Kersters et al. (1996)	D16144
	Pseudomonas radiora	*Methylobacterium radiotolerans*	Kersters et al. (1996)	D3227
	Pseudomonas rhodos	*Methylobacterium rhodinum*	Kersters et al. (1996)	D32229
	Pseudomonas riboflavina'	*Devosia riboflavina*	Kersters et al. (1996)	D49423
	Pseudomonas vesicularis	*Brevundimonas vesicularis*	Kersters et al. (1996)	AB021414
β	*Pseudomonas acidovorans*	*Comamonas acidovorans*	Kersters et al. (1996)	AB021417
	Pseudomonas andropogonis	*Burkholderia andropogonis*	Kersters et al. (1996)	X67037
	Pseudomonas antimicrobica	*Burkholderia* rRNA lineage	Anzai et al. (2000)	AB021384
	Pseudomonas avenae subsp. *avenae*	*Acidovorax avenae* subsp. *Avenae*	Kersters et al. (1996)	AF078759
	Pseudomonas butanovora	*Thauera* rRNA lineage	Anzai et al. (2000)	AB021377
	Pseudomonas caryophylli	*Burkholderia caryophylli*	Kersters et al. (1996)	AB021423
	Pseudomonas cattleyae	*Acidovorax avenae* subsp. *Cattleyae*	Kersters et al. (1996)	AF078762
	Pseudomonas cepacia	*Burkholderia cepacia*	Kersters et al. (1996)	M22518
	Pseudomonas cocovenenans	*Burkholderia cocovenenans*	Kersters et al. (1996)	AB021389
	Pseudomonas delafieldii	*Acidovorax delafieldii*	Kersters et al. (1996)	AF078764
	Pseudomonas facilis	*Acidovorax facilis*	Kersters et al. (1996)	AF078765
	Pseudomonas flava	*Hydrogenophaga flava*	Kersters et al. (1996)	AB021420
	Pseudomonas gladioli	*Burkholderia gladioli*	Kersters et al. (1996)	X67038
	Pseudomonas glathei	*Burkholderia glathei*	Vandamme et al. (1997) Viallard et al. (1998)	AB021374
	Pseudomonas glumae	*Burkholderia glumae*	Kersters et al. (1996)	AB021366
	Pseudomonas huttiensis	*Herbaspirillum* rRNA lineage	Anzai et al. (2000)	U96931
	Pseudomonas indigofera	*Vogesella indigofera*	Grimes et al. (1997)	AB021385
	Pseudomonas lanceolata	*Comamonadaceae* rRNA lineage	Anzai et al. (2000)	AB021390
	Pseudomonas lemoignei	*Burkholderia* group rRNA lineage	Anzai et al. (2000)	AB021375
	Pseudomonas mallei	*Burkholderia mallei*	Kersters et al. (1996)	1680504
	Pseudomonas mephitica	*Janthinobacterium* rRNA lineage	Anzai et al. (2000)	AB021388
	Pseudomonas mixta	*Telluria mixta*	Kersters et al. (1996)	X65589
	Pseudomonas palleronii	*Hydrogenophaga palleronii*	Kersters et al. (1996)	AF019073
	Pseudomonas phenazinium	*Burkholderia phenazinium*	Viallard et al. (1998)	AB021394
	Pseudomonas pickettii	*Ralstonia pickettii*	Kersters et al. (1996)	1680507

[a] Suclasses of Proteobacteria according to Woese, 1987, Stackebrandt et al., 1988 and Kersters et al., 1996.

Table 2.1. continued.

β cont.	*Pseudomonas plantarii*	*Burkholderia plantarii*	Kersters et al. (1996)	U96933
	Pseudomonas pseudoalcaligenes subsp. *citrulli*	*Acidovorax avenae* subsp. *Citrulli*	Kersters et al. (1996)	AF078761
	Pseudomonas pseudoalcaligenes subsp. *konjaci*	*Acidovorax konjaci*	Kersters et al. (1996)	AF078760
	Pseudomonas pseudoflava	*Hydrogenophaga pseudoflava*	Kersters et al. (1996)	AF078770
	Pseudomonas pseudomallei	*Burkholderia pseudomallei*	Kersters et al. (1996)	U91839
	Pseudomonas pyrrocinia	*Burkholderia pyrrocinia*	Vandamme et al., 1997), Viallard et al. (1998)	AB021369
	Pseudomonas rubrilineans	*Acidovorax avenae* subsp. *Avenae*	Kersters et al. (1996)	AB021421
	Pseudomonas rubrisubalbicans	*Herbaspirillum rubrisubalbicans*	Kersters et al. (1996)	AB021424
	Pseudomonas saccharophila	*Matsuebacter*-Proteobacterium rRNA lineage	Anzai et al. (2000)	AB021407
	Pseudomonas solanacearum	*Ralstonia solanacearum*	Kersters et al. (1996)	X67036
	Pseudomonas spinosa	*Hydrogenophaga* rRNA lineage	Anzai et al. (2000)	AB021387
	Pseudomonas syzygii	*Ralstonia* rRNA lineage	Anzai et al. (2000)	AB021403
	Pseudomonas taeniospiralis	*Hydrogenophaga taeniospiralis*	Kersters et al. (1996)	AF078768
	Pseudomonas terrigena	*Comamonas terrigena*	Kersters et al. (1996)	AB021418
	Pseudomonas testosteroni	*Comamonas testosteroni*	Kersters et al. (1996)	M11224
	Pseudomonas woodsii	*Burkholderia* rRNA lineage	Anzai et al. (2000)	AB021422
γ-β	*Pseudomonas beteli*	*Stenotrophomonas* rRNA lineage	Anzai et al. (2000)	AB021406
	Pseudomonas boreopolis	*Xanthomonas-Xylella* rRNA lineage	Anzai et al. (2000)	AB021391
	Pseudomonas cissicola	*Xanthomonas* rRNA lineage	Anzai et al. (2000)	AB021399
	Pseudomonas geniculata	*Stenotrophomonas* rRNA lineage	Anzai et al. (2000)	AB021404
	Pseudomonas hibiscicola	*Stenotrophomonas* rRNA lineage	Anzai et al. (2000)	AB021405
	Pseudomonas maltophilia	*Stenotrophomonas maltophilia*	Kersters et al. (1996)	AB008509
	Pseudomonas pictorum	*Stenotrophomonas-Xanthomonas* rRNA Lineage	Anzai et al. (2000)	AB021392
γ	*Pseudomonas beijerinckii*	*Chromohalobacter* rRNA lineage	Anzai et al. (2000)	AB021386
	Pseudomonas doudoroffiii	*Aeromonas* group-*Vibrionaceae* rRNA Lineage	Anzai et al. (2000)	AB021371
	Pseudomonas elongata	*Microbulbifer* rRNA lineage	Anzai et al. (2000)	AB021368
	Pseudomonas flectens	*Enterobacteriaceae* rRNA lineage	Anzai et al. (2000)	AB021400
	Pseudomonas halodurans	*Halomonas halodurans*	Hebert and Vreeland (1987)	L42619
	Pseudomonas halophila	*Marinobacter-Alcanivorax* rRNA lineage	Anzai et al. (2000)	AB021383

Table 2.1. continued.

γ cont.	*Pseudomonas iners*	*Marinobacterium* rRNA lineage	Anzai et al. (2000)	AB021408
	Pseudomonas marina	*Halomonadaceae* rRNA lineage	Anzai et al. (2000)	M93354
	Pseudomonas nautica	*Marinobacter hydrocarbonoclasticus*	Spröer et al. (1998)	AB021372
	Pseudomonas nigrifaciens	*Pseudoalteromonas nigrifaciens*	Gauthier et al. (1995)	X82146
	Pseudomonas piscicida	*Pseudoalteromonas piscicida*	Gauthier et al. (1995)	X82215
	Pseudomonas stanieri	*Marinobacterium* rRNA lineage	Anzai et al. (2000)	AB021367
(Authentic *Pseudomonas*)	***Pseudomonas aeruginosa* group**			
	Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	Kersters et al. (1996)	Z76651
	Pseudomonas alcaligenes	*Pseudomonas alcaligenes*	Kersters et al. (1996)	D84006
	Pseudomonas anguilliseptica	*Pseudomonas anguilliseptica*	Kersters et al. (1996)	AB021376
	Pseudomonas citronellolis	*Pseudomonas citronellolis*	Kersters et al. (1996)	AB021396
	Pseudomonas flavescens	*Pseudomonas flavescens*	Kersters et al. (1996)	U01916
	Pseudomonas mendocina	*Pseudomonas mendocina*	Kersters et al. (1996)	M59154
	Pseudomonas nitroreducens	*Pseudomonas nitroreducens*	Anzai et al. (1997)	D84021
	Pseudomonas oleovorans	*Pseudomonas oleovorans*	Kersters et al. (1996)	D84018
	Pseudomonas pseudoalcaligenes	*Pseudomonas pseudoalcaligenes*	Kersters et al. (1996)	AB021379
	Pseudomonas resinovorans	*Pseudomonas resinovorans*	Kersters et al. (1996)	AB021373
	Pseudomonas straminae	*Pseudomonas straminae*	Anzai et al. (1997)	D84023
	***Pseudomonas chlororaphis* group**			
	Pseudomonas aurantiaca	*Pseudomonas aurantiaca*	Anzai et al. (2000)	AB021412
	Pseudomonas aureofaciens	*Pseudomonas chlororaphis*	Kersters et al. (1996)	D84008
	Pseudomonas chlororaphis	*Pseudomonas chlororaphis*	Kersters et al. (1996)	D84011
	Pseudomonas fragi	*Pseudomonas fragi*	Kersters et al. (1996)	AB021413
	Pseudomonas lundensis	*Pseudomonas lundensis*	Kersters et al. (1996)	AB021395
	Pseudomonas taetrolens	*Pseudomonas taetrolens*	Kersters et al. (1996)	D84027
	***Pseudomonas fluorescens* group**			
	Pseudomonas azotoformans	*Pseudomonas azotoformans*	Anzai et al. (1997)	D84009
	Pseudomonas cedrella	*Pseudomonas cedrella*	Anzai et al. (2000)	AF064461
	Pseudomonas corrugata	*Pseudomonas corrugata*	Kersters et al. (1996)	D84012
	Pseudomonas fluorescens	*Pseudomonas fluorescens*	Kersters et al. (1996)	D84013
	Pseudomonas gessardii	*Pseudomonas gessardii*	Anzai et al. (2000)	AF074384
	Pseudomonas libaniensis	*Pseudomonas libaniensis*	Anzai et al. (2000)	AF057645
	Pseudomonas mandelii	*Pseudomonas mandelii*	Anzai et al. (2000)	AF058286
	Pseudomonas marginalis	*Pseudomonas marginalis*	Kersters et al. (1996)	AB021401
	Pseudomonas migulae	*Pseudomonas migulae*	Anzai et al. (2000)	AF074383
	Pseudomonas mucidolens	*Pseudomonas mucidolens*	Kersters et al. (1996)	D84017
	Pseudomonas orientalis	*Pseudomonas orientalis*	Anzai et al. (2000)	AF064457
	Pseudomonas rhodesiae	*Pseudomonas rhodesiae*	Anzai et al. (2000)	AB021410
	Pseudomonas synxantha	*Pseudomonas synxantha*	Kersters et al. (1996)	D84025
	Pseudomonas tolaasii	*Pseudomonas tolaasii*	Kersters et al. (1996)	D84028
	Pseudomonas veronii	*Pseudomonas veronii*	Anzai et al. (2000)	AB021411
	***Pseudomonas pertucinogena* group**			
	Pseudomonas denitrificans	*Pseudomonas denitrificans*	Anzai et al. (2000)	AB021419
	Pseudomonas pertucinogena	*Pseudomonas pertucinogena*	Anzai et al. (2000)	AB021380
	***Pseudomonas putida* group**			
	Pseudomonas mosselii	*Pseudomonas mosselii*	Anzai et al. (2000)	AF072688
	Pseudomonas fulva	*Pseudomonas fulva*	Anzai et al. (1997)	D84015
	Pseudomonas monteilii	*Pseudomonas monteilii*	Anzai et al. (2000)	AB021409
	Pseudomonas oryzihabitans	*Pseudomonas oryzihabitans*	Anzai et al. (1997)	D84004

Table 2.1. continued.

	***Pseudomonas putida* group** (cont.)			
	Pseudomonas plecoglossicida	*Pseudomonas plecoglossicida*	Anzai et al. (2000)	AB009457
	Pseudomonas putida	*Pseudomonas putida*	Kersters et al. (1996)	D84020
	***Pseudomonas stutzeri* group**			
	Pseudomonas balearica	*Pseudomonas balearica*	Kersters et al. (1996)	U26418
	Pseudomonas luteola	*Pseudomonas luteola*	Anzai et al. (1997)	D84002
(Authentic *Pseudomonas*) cont.	*Pseudomonas stutzeri*	*Pseudomonas stutzeri*	Kersters et al. (1996)	U26262
	***Pseudomonas syringae* group**			
	Pseudomonas amygdali	*Pseudomonas amygdali*	Kersters et al. (1996)	D84007
	Pseudomonas avellanae	*Pseudomonas avellanae*	Kersters et al. (1996)	U49384
	Pseudomonas caricapapayae	*Pseudomonas caricapapayae*	Kersters et al. (1996)	D84010
	Pseudomonas cichorii	*Pseudomonas cichorii*	Kersters et al. (1996)	AB021398
	Pseudomonas coronafaciens	*Pseudomonas syringae*	Kersters et al. (1996)	Z76660
	Pseudomonas ficuserectae	*Pseudomonas ficuserectae*	Kersters et al. (1996)	AB021378
	Pseudomonas meliae	*Pseudomonas meliae*	Kersters et al. (1996)	AB021382
	Pseudomonas savastanoi	*Pseudomonas savastanoi*	Kersters et al. (1996)	AB021402
	Pseudomonas syringae	*Pseudomonas syringae*	Kersters et al. (1996)	D84026
	Pseudomonas viridiflava	*Pseudomonas viridiflava*	Kersters et al. (1996)	Z76671
Ungrouped	*Pseudomonas agarici*	*Pseudomonas agarici*	Kersters et al. (1996)	D84005
	Pseudomonas asplenii	*Pseudomonas asplenii*	Kersters et al. (1996)	AB021397
	Pseudomonas fuscovaginae	*Pseudomonas fuscovaginae*	Kersters et al. (1996)	AB021381
	Pseudomonas jessenii	*Pseudomonas jessenii*	Anzai et al. (2000)	AF068259

The goal of phenotypic classification is to create clustered groups of strains on the basis of their overall phenotypic similarity. Phenotypic data include single-character data and multiple-character data. Single-character biochemical and physiological tests describe a small component of the total bacterial phenotype and include staining reactions, cell and flagellar morphology and pigment production. Single-character analyses produce overly simple and inaccurate classifications because only a small portion of the bacterial phenotype is represented. Despite this many taxa (i.e. *Cyanobacteria*) based on single-character data have been supported by more comprehensive analyses (Lehtimaki et al., 2000). Multiple-character tests have the advantage that large components of the phenotype can be examined simultaneously and include such chemotaxonomic tests as cell wall composition, fatty acid and protein profiling, and biochemical tests such as carbon substrate utilization profiling.

Phenotypic characterization can be considered to represent the end products of evolutionary relationships. Since prokaryotes are asexually reproducing organisms, hypothetically vertical inheritance should predominate, but some bacteria with highly related phenotypes have phylogenetic relationships that can no longer be ascertained. That is they are polyphyletic meaning that bacteria highly related based on phenotypic characterization can come from different evolutionary backgrounds. Phenotypic incongruence is largely the results of gene loss, gain, modification and lateral gene transfer (Spiers et al., 2000).

2.3.1 Fatty acid methyl ester (FAME) profiles

The fatty acid composition of microbial cells is a multiple-character phenotypic test that has been successfully used to identify and characterize both individual isolates and microbial communities associated with the rhizosphere (Welch, 1991; Rainey et al., 1994, Lilley et al., 1996, Misko and Germida, 2002). Comparison of FAME profiles obtained under similar environmental conditions is highly reproducible (Haack et al., 1994; Cavigelli et al., 1995). However, Haack et al. (1994) suggests that changes in environmental conditions, such as media, temperature, pH and oxygen tension, effects the reproducibility of the obtained FAME data. To obtain a stable and reproducible cellular fatty acid profile, Sasser (2006) recommends careful regulation of growth conditions. Using strictly standardized growth conditions, Bertone et al. (1996) reported a consistently reproducible profile both qualitatively and quantitatively.

Carboxylic acids with long hydrocarbon chains are the basic constituents of lipids among which are the fatty acids. The fatty acids found in bacteria typically range from C12 to C35 with C11-C24 providing the greatest information (Dinel et al., 1990, Thompson et al., 1999). Diversity in fatty acids such as chain length, position of the

double bonds and substituted groups make them valuable biomarkers. Long chain fatty acids can be extracted from bacterial isolates and the types and amounts present are fingerprints of bacterial species. Using the fatty acid compositions, bacteria can be identified by comparison to the MIDI Microbial Identification System (MIDI Inc., Newark, DE) database. In addition, numerical analysis of the fatty acid composition data can be conducted to determine the extent of similarity between isolates. Fatty acid analysis involves saponification of the cells and methylation of the fatty acids, which are extracted and injected into a gas chromatograph. The gas chromatograph separates, identifies and quantifies the fatty acids present in the sample. The resulting profile is compared to those in a database library of known reference organisms (Smibert and Krieg, 1994).

2.3.2 Carbon substrate utilization

The pattern of carbon substrates utilized by microbial cells is a multiple-character phenotypic test that has been successfully used in three ways: to identify bacteria, to characterize the metabolic properties of bacterial isolates and to characterize the metabolic properties of microbial communities (Garland and Mills, 1991; Barnett et al., 1999). Multiple assays of carbon substrate utilization are tested using the commercially available Biolog™ microtiter plates. Each plate contains 95 different carbon sources, with each well containing a different carbon source and tetrazolium violet dye as a redox indicator. Following inoculation and incubation, utilization of the carbon substrate is quantified by well colour development. Utilization of the carbon substrate causes the reduction of the indicator dye and a change in well colour from clear to purple. This method of assessing the functional diversity of bacteria is potentially more useful to microbial ecologists as it provides insight into the roles of microorganisms in the environment (Zak et al., 1994).

However, Garland and Mills (1991) and Winding (1993) both found that variation in responses between samples was not necessarily a function of structural differences in the communities. Instead, both noted a strong correlation between inoculum cell density and the rate of color development. Moreover, both groups found that color development appeared to depend on cell growth in substrate-containing wells, so that fast-growing organisms dominated the observed response. However, Haack et al. (1995) reported high reproducibility for carbon substrate utilization profiles obtained from simple microbial communities of equal inoculum densities.

2.3.3 Extracellular enzyme production

The production of extracellular enzymes within the rhizosphere is necessary for the degradation of both plant and microbial biomass and the degradation of exudates released by the plants. Enzymes commonly found in the rhizosphere are those that degrade biopolymers present in plant and microbial biomass, such as cellulases, xylanases, mannanases and amylases that degrade cellulose, xylan, mannose (constituent of hemicellulose) and starch, respectively. The activity of these enzymes results in the release of carbon that can be transformed into a bioavailable form (Bolton et al., 1993). Other extracellular enzymes important in rhizosphere functioning are protease, urease, nitrate- and nitrite reductase, and acid- and alkaline phosphotase. Protease degrades proteins, such as casein, that are found in plant and microbial cells. Urease is responsible for the breakdown of urea fertilizers into bioavailable forms of nitrogen. Nitrite- and nitrate-reductase enable some *Pseudomonas spp.* to undergo denitrification under oxygen limiting conditions (mentioned above). Acid- and alkaline-phosphotase are responsible for the breakdown of inorganic phosphorous to a bioavailable organic form that can be used by plants and bacteria. Overall, these enzymes are important for the cycling of

carbon, nitrogen and phosphorous within the rhizosphere. Tests for their production are single-character tests that do not provide large contributions to their overall taxonomy, but are very important in determining environmental functioning.

2.3.4 Antibiotic resistance

Many bacterial species are resistant to a number of antibacterial agents. Antibiotic and drug susceptibility may have important practical implications, especially if the organism is pathogenic and is occasionally included in the description of a new species. As mentioned previously, MGEs are important components of the genetic structure of pseudomonads. A large portion of antibiotic resistance such as tetracycline, gentamicin, chloramphenicol and streptomycin are determined by plasmids (R plasmids). The mechanisms of resistance in *Pseudomonas spp*. are in general similar to those found in enteric and in some cases may be transmissible. Antibiotic resistance screening is a single-character test, but provides information into the functioning and gene exchange of these bacteria.

2.4 Phylogenetic classification and characterization of bacteria

Phylogenetics is a field of systematics that examines the evolutionary relationships between living organisms. It includes the study of these relationships, in an effort to clarify taxonomic classifications and the study of the causes behind such relationships. The evolutionary history inferred from such analyses is usually represented as tree-like diagrams that depict an estimated degree of the evolutionary relationships among molecules (i.e. DNA), organisms or both (Brinkman and Leipe, 2001). Cladistics is the means of inferring or hypothesizing relationships among organisms. Phylogenetics is occasionally used synonymously with cladistics because of the word “clade,” a set of organisms that descend from a single ancestor, which is derived from the Greek word for

branch. The main idea behind cladistics is that members of a group or clade are monophyletic in that they share a common evolutionary history and are more related to each other than to members of other clades. Cladistic analysis is usually performed by comparing multiple characters such as base pairs in DNA or amino acids in proteins. However, there are three basic assumptions in cladistical analyses: (i) every group of organisms is related by descent from a common ancestor (fundamental theory of evolution), (ii) there is a bifurcating pattern of cladogenesis and (iii) changes in characteristics occurs in lineages over time (Brinkman and Leipe, 2001). The possibility of creating one all-purpose phylogenetic analysis recipe is unrealistic (Hillis et al., 1993). Ultimately, the structure and size of the data set will determine the reliability and practicality of the numerous phylogenetic algorithms, procedures and computer programs that are available. There are advantages and disadvantages to all methods and programs utilized for phylogenetic analysis and they are often the subject of debate in taxonomic and phylogenetic journals (Hillis et al., 1993; Saitou, 1996; Swofford et al., 1996; Li, 1997). It is molecular DNA phylogeny that will be further discussed.

There are three main programs for phylogenetic analysis, namely maximum parsimony, distance and maximum likelihood methods that include many evolutionary models for sequence variation. A straightforward phylogenetic analysis consists of four steps, namely sequence alignment (includes both building the data model and extracting a phylogenetic dataset), determining the substitution model, tree building and tree evaluation.

A sequence alignment reveals which positions in the sequence were conserved and which have diverged from a common ancestor. Progressive sequence alignment is the most common method of aligning sequences. The steps include: (i) performing pair-

wise alignments of all sequences, (ii) using the alignment scores to produce a phylogenetic guide tree and (iii) aligning the most closely related pair of sequences first and then sequentially adding more distantly related sequences into the alignment based on the guide tree. For a comprehensive review of multiple sequence alignments refer to Mount (2001). Each column of a multiple sequence alignment is assumed to correspond to an individual site that has evolved according to the observed sequence variation and each position evolves independently of each other.

To determine the appropriate substitution model to analyze a multiple sequence alignment, we must first examine the concept of evolutionary trees. Each sequence in an alignment is referred to as a taxon, defined as a distinct unit on the tree with each node on a tree representing a splitting of the evolutionary path of that gene into two different species (assuming a bifurcating pattern of evolution). The length of the branch to the next node represents the number of sequence changes that occurred prior to the next separation and is inferred by the amount of sequence changes. Some types of phylogenetic analyses assume a uniform rate of mutation (molecular clock hypothesis), therefore equal branch lengths would represent each taxon from the node of separation (Li and Graur, 1991; Li, 1997). The molecular clock hypothesis is most suitable for closely related species (Mount, 2001). Analyses that do not assume equal rates of mutation will display different branch lengths on the evolutionary tree. Assuming the molecular clock hypothesis holds it is also possible to predict a root. A root is defined by a basal node that is the common ancestor of all the sequences. A root can also be defined by including a taxon that branched off earlier than the taxa under study. In contrast, the unrooted tree does not reveal the location of an earlier ancestor and does not need to follow the molecular clock hypothesis. Given these considerations a suitable method to

analyze the phylogenetic data set can be chosen (Figure 2.1). Maximum parsimony predicts the evolutionary tree or trees that minimize the number of steps required to generate the variations between sequences. The best results are obtained for maximum parsimony methods when the amount of variation between all pairs of sequences is similar and the amount of variation is small. Because the maximum parsimony method tries to fit all possible trees to the data set, the method is not suitable for more than 11-12 sequences because there are too many trees to test (Mount, 2001).

Maximum likelihood methods use probability calculations to find a tree that best accounts for the variation in a set of sequences. This method is similar to maximum parsimony methods in that analysis is performed on each column of a multiple sequence alignment. Just as with the parsimony methods all possible trees are considered, therefore this method is only feasible when small numbers of sequences are being analyzed. It calculates the trees with the least number of changes as being the most likely. However, trees with variations in mutation rates can be evaluated using evolutionary models such as the Jukes-Cantor and Kimura models (Kimura, 1980; Li and Graur, 1991; Li, 1997) that allow variations in base composition. As a result, this method is particularly useful when analyzing distantly related organisms.

Distance methods employ the number of changes between each pair in a group of sequences to produce a phylogenetic tree. The pair of sequences that has the fewest number of sequence changes is termed as 'neighbours'. On a phylogenetic tree, these sequences share a node or common ancestor that they branch from. The goal of this method is to identify a tree that positions the neighbours correctly and has branch lengths that represent the data set. Like maximum likelihood methods, this method has the

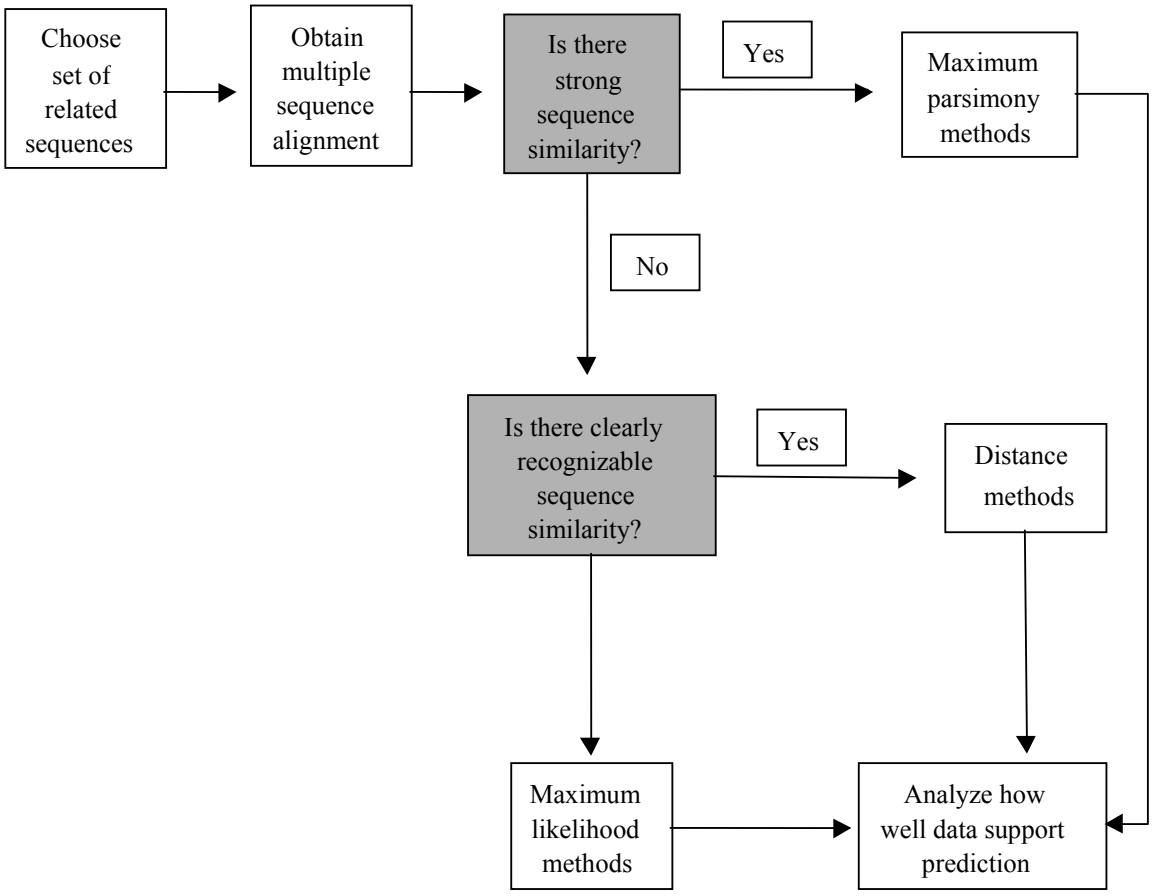

Figure 2.1. Flowchart of methodologies utilized to conduct phylogenetic analysis from a multiple sequence alignment (adapted from Mount, 2001).

advantage of using evolutionary models to allow variations in base composition. The inclusion of an outgroup with trees generated by the distance methods can assist with localization of a tree root without assuming the molecular clock hypothesis (Swofford et al., 1996). Distance methods have been reported to be better predictors than parsimony methods when branch length (mutation rates) may vary (Jin & Nei, 1990). An advantage of the distance methods is that they may be used when analyzing a large number of sequences.

Once a suitable alignment and tree are generated, the prediction should be analyzed to determine how well it represents the original data set. To test the predicted phylogeny, bootstrapping may be utilized. Bootstrapping creates random changes within a multiple sequence alignment to determine the effect of small changes in the alignment on the overall tree topology (Felsenstein, 1988). For branches in the predicted tree topology to be significant, the re-sampled data set should predict the same branches greater than 70% of the time. In addition, it is recommended by Mount (2001) to use at least two of the above methods, if possible, for phylogenetic analysis.

Several areas of the bacterial genome have been used for the inference of phylogenetic relationships. The regions of DNA most commonly used are the 16S rRNA gene, the 16S-23S interspacer region, the *gyr*B gene, the *rpo*D gene and the *cpn*60 gene (Anzai et al., 2000; Yamamoto et al., 2000; Dobson et al., 2002; Hill et al., 2002; Hill et al., 2004). Their utility as phylogenetic markers arises because they encode essential cellular functions. Comprising part of the 'informational' genes of the organisms they are components of complex systems and are less likely to be affected by lateral gene transfer (Jain et al., 1999).

2.4.1 16S rRNA gene

Phenotypic classification of *Pseudomonas spp*. has proved difficult as several members of the genus exhibit similar characteristics. Increasing interest has been placed on gene sequencing as a complement to DNA-DNA hybridizations (which do not allow for phylogenetic inferences) that allows the direct assessment of evolutionary relationships. The 16S rRNA gene has been used successfully for classifications as it is ubiquitous to all prokaryotes and is less likely to be influenced by lateral gene transfer, being an 'informational' gene (Olsen et al. 1986; Woese et al. 1990; Jain et al., 1999; Cole et al. 2003). The use of 16S rRNA sequencing has aided the taxonomy of the genus *Pseudomonas*, but identification of species is often very difficult. Even the finest phenotypic systems (i.e. Biotype-100 strips) cannot resolve species within the *P. fluorescens*, *P. putida* or *P. syringae* complexes (Grimont et al., 1996; Bossis et al., 2000). The use of the 16S rRNA gene as a phylogenetic marker has been criticized, as it assumes one molecule reflects the evolution of an organism and the rate of evolution of the 16S rRNA gene is not sufficient to permit resolution of intrageneric relationships (Yamamoto et al., 2000). Despite this, there remains widespread confidence in 16S rRNA based phylogenetic inferences (Vandamme et al., 1996; Ludwig & Schleifer, 1999).

2.4.2 *cpn*60 gene

Given the limitations posed by 16S rRNA sequence analysis and the impact of lateral gene transfer on genome evolution (Gogarten et al., 2002; Boucher et al. 2003), it is important that our view of taxonomy and phylogenetics be informed by more than one target. Hemmingsen et al. (1988) found that the type I chaperonins were ubiquitous among bacteria and Archaea (Klunker et al., 2003). The type I chaperonins, encoded by the *cpn*60 gene, are also referred to as "GroEL", "MopA" and "Hsp60" in the literature.

Cpn60 has been reported to exhibit multiple cellular functions such as intracellular signaling, but its primary function appears to be as a molecular chaperone for post-translational protein folding and protein complex assembly (Maguire et al., 2002). The discriminatory ability of the *cpn*60 gene sequence to resolve closely related organisms has been established for *Bartonella* and *Streptococcus* and it has been concluded to contain more phylogenetically informative data than 16S rRNA gene sequences (Marston et al. 1999; Brousseau et al., 2001). Like 16S rRNA, the *cpn*60 is an "informational" gene, thus less likely to be subject to lateral gene transfer. Unlike the 16S rRNA gene, there is usually only a single copy of the *cpn*60 gene in prokaryotes and it is not subject to operon heterogeneity (Hill et al., 2002). Overall this suggests that for a comprehensive phylogenetic analysis more than one target is required to overcome the pitfall of analyses conducted using single gene targets.

2.5 Diversity among pseudomonads

The members of the genus *Pseudomonas* are an exceptionally diverse group of organisms exhibiting high levels of physiological and genetic diversity even within species. Their ability to colonize a wide range of habitats suggests a remarkable degree of physiological and genetic diversity. Several papers document microbial diversity at the community and at the organism level, but few examine the causes or ecological significance of this diversity. Diversity arises and is maintained through the interaction between ecological and genetic factors. The ultimate cause of diversification is mutation, but the variation generated by both mutation and recombination (includes lateral gene transfer) is sorted and shaped by selective pressure and genetic drift (Spiers et al., 2000). Genetic factors are the ultimate determinants of patterns of diversity for without genetic variation there can be no evolution or divergence. Mutation and selection alone are

powerful evolutionary forces, but nevertheless the ubiquitous nature of *Pseudomonas spp.* leads many to wonder if they are inherently more evolvable or if lateral gene transfer is largely the cause of their large diversity.

2.5.1 Mutation

Mutation is the ultimate source for the genetic variation required for adaptive divergence of *Pseudomonas spp*. Increasing its mutation rate can enhance the adaptive divergence or evolvability of an organism. It has been postulated that *Pseudomonas spp*. have developed mechanisms to increase their mutation rate when under stress (Spiers et al, 2000). Although there is no direct evidence to support this hypothesis, strains have been identified with elevated mutation rates. For example, *P. aeruginosa* strains with elevated mutation rates (defective mismatch-repair genes) have been isolated from the lungs of patients with cystic fibrosis that have had long-term infections (Oliver et al., 2000). Despite their high mutation rates, there is no evidence that the strains themselves developed a mechanism to enhance their evolvability. Under stress conditions, bacteria have been reported to have elevated mutation rates because of the SOS response and the reduced ability to deal with DNA-damaging free radicals generated by metabolism (Friedberg et al., 1995).

Contingency loci, present in some pathogenic bacteria, are the best example of evolvable DNA sequences (Moxon et al., 1994). Contingency loci generate substantial phenotypic diversity in a rapid time period because they are characterized by short DNA repeats that are prone to slippage and inherently unstable. Polymorphisms in *Pseudomonas* clones, similar to antigenic variation in pathogenic bacteria, have been exhibited by some pseudomonads. For example, the rapid conversion of some *P.aeruginosa* colonies from mucoid to non-mucoid mimics control by a contingency

locus, but no such mechanism has been found (Govan & Deretic, 1996). Analysis of the *P.aeruginosa* PAO1 genome has revealed no contingency loci and assuming they are typical of other pseudomonads, it suggests that they contain no mechanisms to enhance their mutation rate to better their adaptive divergence (Spiers et al., 2000).

2.5.2 Recombination

The impact of recombination (including lateral gene transfer) was emphasized by Lawrence and Ochman (1998) who found that all phenotypic characteristics distinguishing *E.coli* from *S. enterica* were encoded by horizontally transferred genes. In addition, Garcia-Vallvé et al. (2000) observed that 1.6-14.5% of the total microbial genome resulted from horizontally transferred genes with the highest percentages found among the archaea and non-pathogenic bacteria. The analysis of whole-genome sequences has emphasized the extent to which bacterial genomes are dynamic chimeras. The acquisition of genes and MGEs (plasmids, transposons, integrons and phages) by lateral gene transfer is a pervasive force in bacterial evolution (Ochman et al., 2000). Several studies have reported the occurrence of recombination within pseudomonads (Haubold & Rainey, 1996; Kiewitz & Tummler, 2000). Despite recombination events, a clonal framework remains even from isolates separated spatially and temporally. Nevertheless, the ability of pseudomonads to exploit new environments and to respond to environmental stress is most easily explained by the acquisition of new genes by MGEs. The presence of large, broad host range plasmids is commonplace among *Pseudomonas spp.* (Sesma et al., 2000; Thomas, 2000). Alfano et al. (2000) reported the presence of tracts of DNA with atypical codon bias indicative of recent gene acquisition in *Pseudomonas syringae*.

In addition, intragenomic recombination appears to be an important factor in *Pseudomonas* evolution. Kiewitz et al. (2000) found intragenomic recombination accounted for a large portion of the diversity in macrorestriction fragment patterns observed among closely related *Pseudomonas* isolates.

2.5.2.1 Mobile genetic elements

Bacterial genomes are highly dynamic, accumulating ecologically useful sequences by lateral gene transfer and losing genes with no ecologically useful function by deletion. Lateral transfer of genetic material is mediated by MGEs. MGEs can be distinguished from chromosomes by their reproductive strategies. For example, MGEs can be transferred horizontally and then passed vertically to daughter cells, whereas chromosomes are usually only transferred vertically. MGEs include plasmids, transposons, integrons, and bacteriophages.

2.5.2.1.1 Plasmids

Plasmids are extrachromosomal DNA elements that have the ability to self-replicate independently of chromosomal DNA. Plasmids vary widely in size ranging from 1kb (kilobases) to >500kb, and like the chromosome, are negatively supercoiled covalently closed circular DNA elements (Madigan et al., 1997). Only a few of the proteins required for their own replication are plasmid encoded, namely those required for replication initiation at the *ori*V site. The majority of proteins are borrowed from the host, such as DNA polymerases and primases. Many of the characteristics of plasmids are derived from the *ori*V region. These characteristics include the mechanism for replication, copy number, partitioning, and incompatibility (Snyder and Champness, 1997). The physical map of *Pseudomonas putida* plasmid pADPTel is included as an

example of plasmid structure (Figure 2.2). The pADPTel plasmid is the naturally occurring pADP plasmid altered to contain the tellurite resistance genes.

Transfer of plasmid DNA occurs by three separate mechanisms; conjugation, transduction, and transformation. However, the majority of plasmid transfers result from the conjugal transfer of self-transmissible plasmids. Self-transmissible plasmids are capable of transferring themselves to other bacterial cells. Self-transmissible plasmids have a *trans*-acting *tra* region that encodes the products required for transfer, as well as an *ori*T site at which transfer initiates. Moreover, self-transmissible plasmids can mobilize other plasmids that contain the same *ori*T sequence that cannot initiate transfer themselves (mobilizable plasmids) (Snyder and Champness, 1997).

Plasmids can encode a few to hundreds of different proteins, but rarely encode gene products essential for growth such as RNA polymerases and ribosomal subunits. Instead, many bacterial plasmids contain genes functional in antibiotic resistance, virulence for animal or plant hosts, or the anabolism/catabolism of diverse compounds (Summers, 1996). Bacteria harbouring plasmids have been isolated from all natural environments, but the principal source has been soil (Sayler, 1990) (Table 2.2). The majority of these plasmid-bearing strains are Gram-negative organisms, such as *Pseudomonas spp*. Plasmids harbored in pseudomonads and several other environmental bacteria are commonly quite large ranging from ca. 50 to 500kb in size, and are present in low copy numbers (1-2 copies per cell) (Lennon and DeCicco, 1991; Campbell et al., 1995b). These plasmids tend to encode complete pathways, often catabolic or virulence associated, and the associated regulatory machinery making them large by necessity. The enormous sizes of these plasmids, and their presence in low numbers has been reported to hinder their endogenous isolation (Williams et al., 1979; Hirai et al., 1982). However,

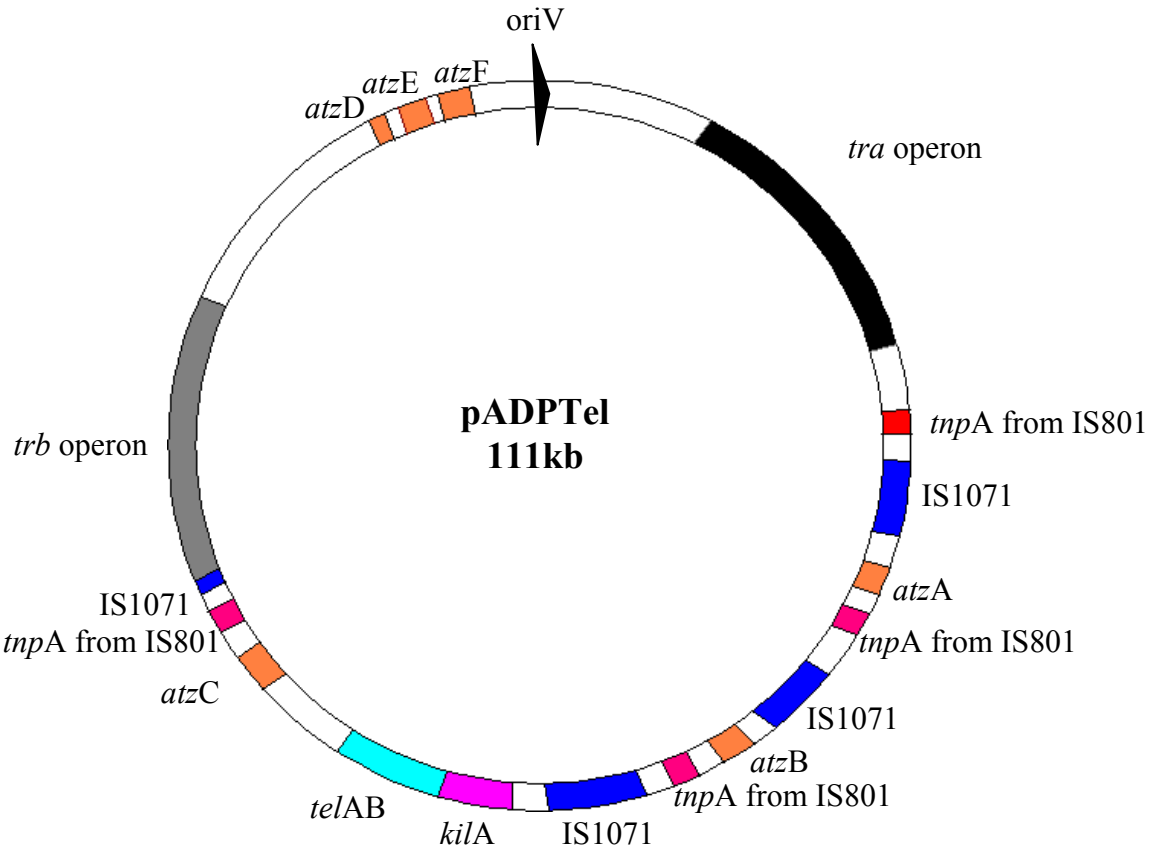

Figure 2.2. Physical circular map of the plasmid, pADPTel. *tnp*, transposase genes, IS, insertion sequences, *atz*, atrazine catabolism genes, *tra* operon, transfer operon, oriV, origin of replication, *kil*A*tel*AB, tellurite resistance operon, *trb* operon, genes encoding mating pair formation apparatus (Hirkala and Germida, 2004).

Table 2.2. Examples of naturally-occurring plasmids found within Gram-negative *Pseudomonas spp*.

Plasmid	Bacterial Strain	Size (kb)	Incompatibility Group	Transmissible	Properties[a]	Reference
pIAA1	*P. syringae*	52	NK[b]	NK	Indole-3-acetic acid (IAA) synthesis	Palm, 1989
PRP4	*P. aeruginosa*	57	P1	+	Cb, Km, Tc	Jacoby, 1977
pRC10	*P. cepacia*	81	NK	+	2,4-dichlorophenoxy-acetate catabolism	Vandenburgh, 1981
PCPP519	*Pseudomonas sp.*	83	Type pPT23A	+	Sm	Huang, 1999
PMAC1	*P. syringae*	83	NK	NK	Coronatine production	Sato, 1989
pNPL1	*P. putida*	87	P9	NK	Napthalene catabolism	Boronin, 1977
PADP-1	*P. putida*	96	NK	+	Atrazine catabolism	DeSouza, 1998
pAC25	*P. putida* AC858	117	NK	+	3-chlorobenzoate catabolism	Chatterjee, 1981
pWW0	*P. putida* (arvilla) mt-2	117	P9	+	Toluene catabolism	Williams, 1974
PAV505	*P. savastanoi*	140	Type pPT23A	+	Curing results in loss of pathogenicity	Jackson,1997
pPGH1	*P. putida* H	200	NK	+	Phenol catabolism	Herrmann, 1987
pMG2	*P. aeruginosa*	400	P2	NK	Gm, Sm, Su, Hg, Pm, UV	Lehrbach, 1977
PMG1	*P. aeruginosa*	~500	P2	mob[c]	Sm, Su, Gm, Hg	McCombie, 1983
pCAM	*P. putida* PpG1	~500	P2	+	Camphor catabolism	Rheinwald, 1973

[a] Abbreviations for resistances: Cb – carbenicillin, Sm – streptomycin, Km – kanamycin, Tc – tetracycline, Su – sulfonamides, Gm – gentamicin, Hg – mercuric chloride, Pm – phenylmercury acetate, UV – ultraviolet
[b] Not known
[c] Mobilizable

Pedraza and Díaz-Ricci (2002) have successfully modified the extraction method of Eckhardt (1978) to reproducibly isolate large, unstable plasmids. In addition, Götz et al. (1995) and Disqué-Kochem et al. (2001) have reported the successful use of PCR to detect broad-host-range self-transmissible plasmids from environmental samples.

2.5.2.1.2 Transposons

Transposons are discrete DNA segments flanked by inverted repeats able to move even in the absence of genetic homology between the donor and target sites (Berg and Howe, 1989). Movement of a transposon is called transposition, and is facilitated by enzymes called transposases, which are encoded by the transposon itself. There are several different types of transposons; the smaller ones are ca. 1000bp long and only encode the genes for transposition, whereas the larger ones may also contain one or more other genes, such as those for antibiotic resistance and biodegradation of organic compounds. A distinguishing factor of all bacterial transposons is the presence of inverted nucleotide sequence repeats (8-40bp) flanking a unique DNA sequence (Tan, 1999). There are three classes of transposons that are classified according to their genetic organization, DNA sequence homologies, and mechanistic properties (Figure 2.3) (Grindley and Reed, 1985). Class I transposons include the insertion sequences (IS) and composite transposons that contain an intervening sequence with flanking IS (Syvanen, 1988). Transposons belonging to class II or Tn3-like transposons have shorter (<50bp) inverted repeats sequences, and involve a transposase (TnpA) and a resolvase (TnpR) in a two-step transpositions process that creates a 5bp duplication of the target sequence (Sherratt, 1989; Grinsted et al., 1990). Class III transposons are the transposing bacteriophages, such as Mu. The majority of transposons isolated from environmental bacteria are classified as belonging to class I or II (Tan, 1999).

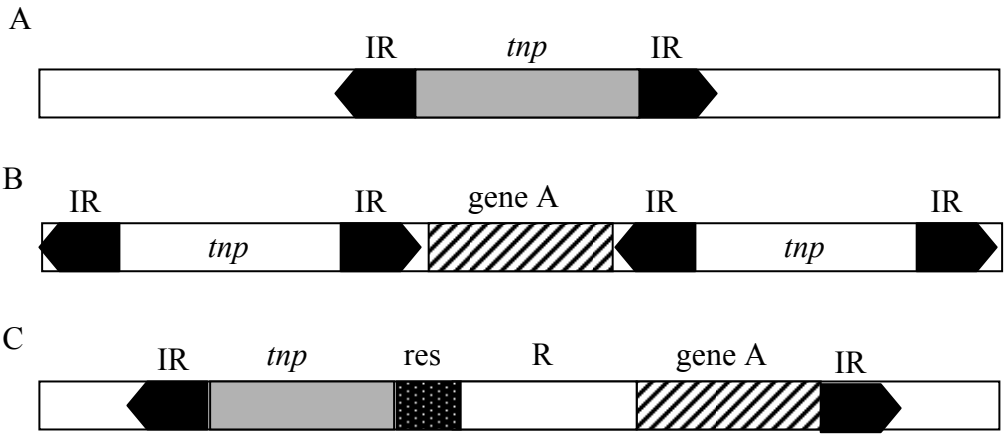

Figure 2.3. General structure of transposons: (A) insertion sequences, (B) composite transposons and (C) non-composite transposons. *tnp*, transposase gene, IR, inverted repeats, res, recombination site and R, resolvase (adapted from Snyder and Champness, 1997).

Transposons mediate gene flow by entering other bacteria during the transfer of promiscuous plasmids or via transducing phage. There is growing evidence that transposition occurs frequently in the environment. For example, antibiotic resistance genes with >95% DNA sequence identity have been found in terrestrial bacteria separated geographically. In addition, recent studies report the discovery of several class I and II type transposons within terrestrial bacteria (Assinder andWilliams, 1990; Salyers et al., 1998; Smalla et al., 2000).

Transposons and insertion sequences are known to be highly mutagenic elements, and also are responsible for the mobilization of xenobiotic degradation genes (Mahillon and Chandler, 1988; Tan, 1999). Examination of transposable elements could result in the discovery of new genes with beneficial functions, and in a better understanding of gene flow in the environment. Schneider et al. (2000) successfully isolated and sequenced transposable elements from several Gram-negative bacteria using plasmid pGBG1 (accession # AJ277653). Plasmid pGBG1 contains a mutagenic target composed of a silent *tet*A (tetracycline resistant phenotype) gene under the control of the pR promoter of bacteriophage lambda, which is repressed by the cI repressor. Spontaneous insertion or deletion of transposable elements that inactivate cI or eliminate its binding site will trigger the expression of *tet*A. Since the sequence of the plasmid is known, the transposable elements captured by the plasmid can be sequenced by generating primers to the plasmid. The use of this technique not only detects transposable elements, but also results in the isolation of new elements.

2.5.2.1.3 Integrons

Integrons are genetic elements that mediate the integration of genes by a site-specific recombination mechanism. Integrons mobilize genes by incorporating transposons of the

Tn*21* family or by residing on broad-host-range plasmids (Lévesque et al., 1995). Integrons possess two conserved regions separated by a variable region, which includes integrated genes, such as antibiotic resistances (Martinez-Freijo et al., 1998). Integrons are essentially gene acquisition and expression systems. Gene cassettes are acquired by a site-specific recombination reaction catalyzed by the integron encoded integrase, IntI (Collis and Hall, 1992). Site-specific recombination occurs within the variable region at a short imperfect inverted repeat element called the 59-base element (Stokes and Hall, 1989) (Figure 2.4). Expression of the integrated gene cassettes is initiated from common promoters (P1 and P2) resulting in lower efficiency of transcription of more distal genes (Collis and Hall, 1995).

Horizontal gene transfer is mediated by a number of MGEs, including plasmids, transposons, integrons, and transducing bacteriophage. However, most studies have focussed on plasmids or transposons. Since integrons have recently been demonstrated to occur in the chromosomes of diverse bacterial species, and integrases have been recovered from environmental samples, Stokes et al. (2001) reasoned that integrons are widespread in natural environments. The dissemination of integrons, and their encoded genes is still unknown. However, polymerase chain reaction (PCR) has been used to recover gene cassettes within integrons, and to detect the presence of integrons within clinical isolates (Martinez-Freijo et al., 1998; Stokes et al., 2001).

2.5.2.1.4 Bacteriophages

Bacteriophages are obligate intracellular parasites of bacteria, and have no independent metabolic activity (Kokjohn and Miller, 1992). They are able to facilitate gene flow by transduction, a process whereby bacterial DNA is packaged within viral particles capable of infecting other bacteria. Transduction is most often mediated by

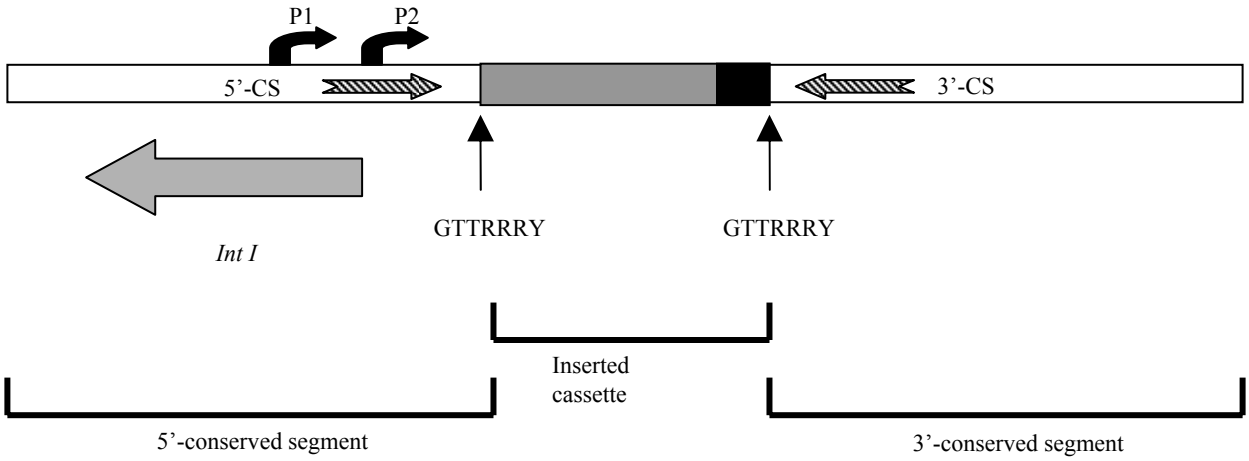

Figure 2.4. General structure of integrons. The location and orientation of the promoters, P1 and P2, are shown. The sequence GTTRRRY is the integron's crossover point for integration for gene cassettes. The binding sites for the 5'-CS/3'-CS primer set are shown. One insert cassette is shown with its associated 59-base element indicated by the black bar. Adapted from Lévesque et al., 1995.

temperate phages (which establish a long-term symbiotic relationships with their hosts, called lysogeny), but can occur by virulent phages. There are two types of transduction; generalized, where any genetic material within the host cell has an equal chance of being transduced, and specialized, where only specific genetic elements are transduced (Morse, 1954; Ozeki & Ikeda, 1968).

Gene flow mediated by bacteriophages has been reported for a variety of environmental bacteria, including *Pseudomonas spp*., *Bacillus spp*., *Rhizobium* s*pp*., and *Erwinia spp*. (Kokjohn & Miller, 1992). Because of the restricted host range of bacteriophages, and the fact that transduction is mediated by an external factor it has been assumed that they contribute minimally to horizontal gene transfer in the environment. The available data suggest that lysogeny is common in nature. Farrand (1989) found many bacteria isolated from plants were lysogenic. Holloway (1969) estimated 100% of natural *P. aeruginosa* isolates were lysogenic, and postulated that many contained prophages. Germida and Khachatourians (1988) and Zeph et al. (1988) and others have observed generalized transduction in soil microcosms between phage P1 and the introduced recipient bacteria, *E. coli*.

2.6 Lateral gene transfer of genetic material in the rhizosphere

Genetic material may be transferred laterally (used synonymously with horizontally) from a donor to a recipient organism by three methods: conjugation, transformation and transduction. Conjugation is the transfer of plasmid DNA by direct cell-to-cell contact. Transposition refers to the transfer of transposable elements and is mediated during the transfer of conjugative plasmids and during the induction of lysogenic phages. Transduction is the process in which donor DNA is packaged into a bacteriophage capsid and introduced into another bacterium by infection by the bacteriophage containing the

donor bacterium's DNA. Transformation is the process by which competent bacterial cells uptake free DNA from the extracellular environment and express the foreign genetic material.

2.6.1 Conjugation

Conjugal transfer of DNA occurs through a sex pilus that grows from the donor cell surface, and interacts with specific sites on the surface of the recipient cell. Self-transmissible plasmids carry the transfer (tra) operon, which encodes all the functions required for conjugation including the formation of the sex pili. Self-transmissible plasmids can integrate into the chromosome leading to the possible transfer of chromosomal genes, and can mediate the transfer of mobilizable plasmids when they coexist in the same cell. The autonomous nature of plasmids, and the fact that they do not require homologous DNA sequences for their stable maintenance in recipient cells makes conjugation an efficient mode of gene transfer (Trevors et al., 1987). In fact, Rensing et al. (2002) suggests that conjugation could possibly be the most important means of gene transfer in the environment. The efficiency of conjugal gene transfer depends on the conjugative plasmid as well as the donor and recipient bacteria, and thus can be influenced by several biotic and abiotic factors.

Some of the environmentally relevant biotic factors are the physiological status of the donor and recipient cells, the properties of the plasmid, the presence of indigenous microorganisms, and the presence or absence of a rhizosphere. Several studies have addressed the dependency of plasmid transfer frequency on the physiological status of the donor and recipient cell populations (Smets et al., 1993; Muela et al., 1994; Sudarshana & Knudsen, 1995; Arana et al., 1997). The general conclusion of these studies is that conjugation frequency is dependent on the physiological status of the donor rather than

the recipient cells. Studies conducted by van Elsas et al. (1987) and Trevors & Oddie (1986) have demonstrated that nutrient amendment stimulates *in situ* conjugal activity and thus indicates the importance of cell physiology in mating pair formation.

The properties of the plasmid such as the expression of the *tra* genes, the incompatibility group, and the type of pili encoded by the plasmid affect the conjugation frequency. Expression of the *tra* operon is highly regulated, and thus increases in transfer rates are observed when the *tra* genes are derepressed (Sayler et al., 1990). The incompatibility group of the plasmid will determine the host range in which the plasmid can be transferred and expressed, for two plasmids belonging to the same incompatibility group are unable to coexist in the same cell. Transfer frequencies of broad-host-range plasmids will thus be greater than those able to inhabit only a small number of hosts (Pukall et al., 1996). Conjugative plasmids can encode for either short, rigid pili or long, flexible pili. Plasmids encoding short, rigid pili exhibit greater transfer frequencies in environmental systems containing high solid to liquid ratios where they avoid the shearing forces of liquid environments (Dröge et al., 1999).

Indigenous microorganisms have been reported to exhibit a negative influence on conjugative gene transfer in terrestrial environments (Top et al., 1990; Neilson et al., 1994). The indigenous microorganisms may interact with the introduced donor organism in a competitive, antagonistic, parasitic or predatory nature. The metabolic activity or the survival of the bacteria is compromised, resulting in decreased transfer frequencies. In natural soil, the transfer frequency was below detectable limits while in sterile soils transfer was observed (Top et al., 1990; Neilson et al., 1994). In a study conducted by Kuikman et al. (1990) the effect of protozoal populations on conjugation frequency was

examined. It was concluded that protozoa did not influence plasmid transfer frequencies in soil.

The presence of plant roots was found to stimulate conjugative gene transfer (van Elsas et al., 1988; Smit et al., 1991). These findings may be attributed to improved nutrient status of the bacterial cells or the vast surface area provided by the root surfaces, which are conducive for conjugation events. Recently, Shwaner and Kroer (2001) have demonstrated that the frequency of conjugal transfer in the rhizosphere changes with different plant species. For example, the highest transfer frequencies were observed in the rhizosphere of pea, followed by the barley and wheat rhizosphere.

Some of the abiotic factors that influence conjugation are temperature, moisture, pH, and clay minerals. The influence of temperature on conjugation frequency has been extensively addressed in such habitats as soil (Richaume et al., 1989; Kinkle and Schmidt, 1991; Lafuente et al., 1996). Overall, a positive correlation between temperature and transfer frequencies has been noted. Maximum transfer frequencies are observed at the optimal growth temperature for the organism, for example when *Escherichia coli* was used as a donor organism the optimal conjugation frequency was observed at 30°C, whereas when *Sinorhizobium fredii* was used as a donor organism the optimal frequency was observed at 20°C (Kinkle and Schmidt, 1991; Lafuente et al., 1996).

Moisture content is an important factor affecting the frequency of plasmid transfer for it influences the availability and distribution of nutrients, the physiological status of the donor and recipient cells, the probability of cell-to-cell contact, and the stability of mating pair formation (Dröge et al., 1999). Variable results have been reported concerning the effect of moisture on conjugal plasmid transfer in soil. Lafuente et al. (1996) reported

optimal transfer frequencies at a water content of 20% (v/w), whereas Richaume et al. (1989) reported optimal transfer frequencies at a water content of 8% (v/w). Thus it is suggested by Dröge et al. (1999) that the optimal moisture content is dependent on the corresponding donor, plasmid, and recipient cell combination.

The influence of pH on plasmid transfer in soil has been addressed by Weinberg & Stotzky (1972), Krasovsky and Stotzky (1987), Richaume et al. (1989), and Lafuente et al. (1996). The general conclusion is that plasmid transfer frequency increases as pH approaches neutrality.

The addition of montmorillonite and bentonite clays has been shown to exert positive effects on plasmid transfer, whereas the addition of kaolinite clays has no effect on plasmid transfer frequency (Weinberg and Stotzky, 1972; Krasovksy and Stotzky, 1987; Richaume et al., 1989). Montmorillonite and bentonite clays have large cation exchange capacities and thus serve to buffer soils against pH changes, and bind soluble organic compounds creating conditions conducive for gene transfer (Stotzky, 1986). They also provide large charged surfaces on which cells are immobilized thus facilitating cell-to-cell contact. Koalinite clay has a lower specific surface area, and cation exchange capacity explaining its lack of effect on gene transfer (Krasovsky and Stotzky, 1987). In general, any parameter that stimulates activity of the introduced donor will also stimulate conjugal plasmid transfer. This is derived from the observation that most factors, which influence transfer frequencies act at the level of cell-to-cell contact and the host cells' physiology.

2.6.2 Transformation

Natural genetic transformation encompasses the active uptake and expression of free exogenous DNA (plasmid and chromosomal). Natural genetic transformation is to be

distinguished from 'artificial' or *in vitro* procedures used to introduce DNA molecules into bacteria. Bacteria are the only organisms capable of natural transformation. Gene transfer by natural transformation can be categorized into six steps; (i) the release of DNA from cells, (ii) the dispersal of the free DNA, (iii) the persistence of the DNA in the environment, (iv) the development of competence for DNA uptake, (v) the interaction of cells with DNA and its subsequent uptake, and (vi) the expression of the acquired genetic material.

Natural transformation is mediated by free DNA released from cells. Free DNA has been detected in aquatic and soil environments. Ogram et al. (1987) reported the isolation of 1μg of purified extracellular DNA per g of sediment. Steffan et al. (1988) suggested that the large amounts of DNA recovered from soil by direct DNA extraction from soil might indicate the presence of extracellular DNA. Extracellular DNA in the environment originates as a result of DNA release from microbial cells. Processes that promote DNA release from cells are dependent on abiotic factors, such as pH and temperature, and biotic factors, such as viral infection and protozoal predation (Lorenz and Wackernagel, 1994).

The passive dissemination of free DNA in the environment determines how far the recipient cell can be separated from the donor cell, for transformation to occur (Leff, et al., 1992). Free nucleic acids introduced into the soil are hydrolyzed at a substantial rate, by DNA-hydrolyzing bacteria (Greaves et al., 1970). However, extracellular DNA is still found in terrestrial environments. Factors that contribute to the persistence of extracellular DNA in soil are the continuous production of DNA by microorganisms, and the adsorption of DNA onto soil minerals (Greaves, 1970). DNA associated with the charged surfaces of silicate materials, like quartz and clay, exhibit increased resistance to

degradation by Dnase I (Paget et al., 1992). In fact, Romanowski et al. (1992) demonstrated that naked DNA introduced into non-sterile soil microcosms persisted for months despite slow continual breakdown.

The competence of cells refers to their ability to take in free DNA from the external environment. Some of the soil bacterial species shown to develop natural competence are *Pseudomonas stutzeri* and related species, *Rhizobium meliloti*, and *Azotobacter vinelandii* (Lorenz and Wackernagel, 1991). Development of a competent state is influenced by several environmental parameters, including nutrient utilization, nutrient limitation, calcium, and pH and temperature. Competence requires that the bacteria are in a metabolically active state therefore, stable nutrients must be available (Dröge et al., 1999). Examination of the effect of nutrient limitation on competence provided variable results. For example, competence was stimulated when cells were starved for one nutrient, but limitation of two or three nutrients exhibited inhibitory effects (Lorenz and Wackernagel, 1994). The presence of calcium in the medium has been shown to play a key role in the competence development of soil microorganisms, including *Azotobacter vinelandii* (Page et al., 1979). The effects of pH and temperature on transformability appear to be species specific. For example, the transformability is the greatest at neutral pH in *A. vinelandii* and *P. stutzeri*, whereas *S. pneumoniae* exhibits the greatest transformability at alkaline pH (Lorenz and Wackernagel, 1994).

The uptake of free DNA by competent cells, and its subsequent expression is limited by the availability of DNA in solid matrices, like soil, and the requirement of homologous sequences in the genome for integration and expression (not required for plasmid DNA). The ratio between target versus non-target DNA sequences on which homologous recombination can occur is very low, thus restoring the genetic barrier to

gene transfer by vastly decreasing the frequency of successful transformation (Kay et al., 2002).

Transformation has been extensively demonstrated in soil environments (reviewed by Lorenz and Wackernagel, 1994). Recently, transformation of transgenic plant DNA to bacteria has been demonstrated under environmental conditions (Nielsen et al., 2000; Kay et al., 2002). These findings stress concerns regarding the fate of engineered genetic elements upon release into the environment, both microorganisms and plants.

2.6.3 Transduction

Transduction is dependent on the interaction of a transducing phage and a host cell that will act as a donor, and the interaction of the transducing particle with an appropriate recipient. Since the initial interaction is thought to be passive, the transduction frequency is dependent on the concentration of donor and recipient organisms, as well as the concentration of phages. The potential for gene transfer by transduction can be inferred from reports on high concentrations of bacteria and phages in terrestrial environments (Germida and Casida, 1983; Campbell et al., 1995a). In addition, transducing phages have been observed in a variety of environmental bacteria, including *Pseudomonas spp*., *Bacillus spp*., *Rhizobium spp*., and *Erwinia spp*. (Kokjohn and Miller, 1992). Ogunseitan et al. (1992) confirmed these observations by demonstrating the wide distribution of transducing phages and prophages among natural *Pseudomonas* populations.

Bacteriophages have a narrow host range, sometimes limited to a specific bacterial species. The limited host range would make transduction seem like an unlikely candidate for promoting gene flow in the environment. However, phages are very common and stable in the environment making them conducive to intraspecific gene transfer (Davison, 1999). In nutrient poor environments, such as soil, phages tend to form lysogenic states

within their host promoting transduction under nutrient stress. In addition, the presence of clay minerals favours host/phage interactions thereby enhancing the transfer frequency (Dröge et al., 1999). Using *P. aeruginosa* phage F116, Kidambi et al. (1994) observed the transduction of plasmid and chromosomal markers on the phylloplane and in soil microcosms.

2.6.4 Transposition

The transfer of transposable elements is mediated during the transfer of conjugative plasmids, and during the induction of lysogenic phages. The majority of transposition events have been inferred from antibiotic resistance. Cooper et al. (1996) provided evidence that transposition occurs in nature between soil and intestinal bacteria by showing that the *erm*G alleles in *Bacillus spp*. and *Bacteroides spp*. were more than 99% identical at the DNA sequence level. In addition, recent studies report the discovery of several class I and II type transposons within terrestrial bacteria (Salyers et al., 1998; Assinder and Williams, 1990; Smalla et al., 2000). Despite the prevalence of transposons among terrestrial bacteria, few studies have examined the transfer of transposable elements in soil or rhizosphere environments. Rather the focus has been on clinical isolates. Since transposition does not require homologous sequences to integrate into the genome, and they are capable of jumping onto other MGE, their influence on gene flow could be quite significant. Transposons (and integrons) are relatively new MGE when considering plasmids and bacteriophages. Considerable research is required to examine the contribution transposons confer on horizontal gene flow in the environment, including the rhizosphere.

In summation, the rhizosphere is a zone of increased microbial activity as a result of plant exudates. It is well documented that this zone of increased microbial activity

affects plant and soil quality and hence affects ecosystem sustainability. The most numerous of the rhizosphere microorganisms are bacteria ranging from 10^6 to 10^9 g^{-1} rhizosphere soil. Of the rhizosphere bacteria, *Pseudomonas spp*. have been reported to dominate the rhizospheres of tomato, flax, sugar beet, rice and canola. *Pseudomonas spp*. are Gram negative flagellated rods that exhibit exceptional phenotypic and genetic diversity. Pseudomonads are important as they contribute to nutrient cycling, to soil remediation of xenobiotic compounds, and are able to promote plant growth. Their large diversity is mediated by mutation and recombination, but their ubiquitous nature raises the question if they are inherently more evolvable or if lateral gene transfer is largely the cause of their large diversity. MGEs are the genetic elements that mediate lateral gene transfer and include plasmids, transposons, integrons and bacteriophages. MGEs are transferred laterally from donor to a recipient organism by three methods: conjugation, transformation and transduction.

In light of the literature, my Ph.D. thesis research initiated a project to examine the variation exhibited between the physiological and the genetic diversity of a set of rhizosphere pseudomonad isolates. I hypothesized that the presence of MGEs within pseudomonad isolates would correlate with discrepancies observed between their phylogenetic and physiological relationships. The main objectives of my study were:

1) To compare the physiological properties and the phylogenetic relationships of these pseudomonad isolates.
2) To examine the diversity of their associated mobile genetic elements (MGEs).

3.0 TAXONOMIC DIVERSITY OF ROOT-ASSOCIATED PSEUDOMONADS

3.1 Introduction

Pseudomonas spp. constitute a large group of highly diverse bacteria found in large numbers in all of the natural environments (terrestrial, freshwater and marine) and can form associations with plants. The many important characteristics of *Pseudomonas spp*. have led to numerous studies examining their taxonomy and diversity. Taxonomic diversity is most often studied on two levels: phenotypic diversity and genetic diversity. The classification and taxonomy of pseudomonads have been based on selective culturing (Sayler et al., 1990), restriction fragment length polymorphism analysis of amplified 16S rRNA genes (Laguerre et al., 1994; Brosch et al., 1996; Porteous et al., 2002), sequence analysis of the *gyr*B, *rpo*D and 16S rRNA genes (Yamamoto & Harayama, 1998; Anzai et al., 2000), DNA and RNA hybridization (Palleroni et al., 1972; Palleroni et al., 1973), protein and fatty acid analyses (Vancanneyt et al., 1996a; Vancanneyt et al., 1996b) and carbon source utilization (Klingler et al., 1992; Grimont et al., 1996). However, identification of *Pseudomonas* isolates to the species delineation is often difficult.

While phenotypic profiling is useful in the classification of variable organisms, bacteria often exhibit similar properties making classification difficult. Analysis of DNA and protein sequences allowed the assessment of phylogenetic relationships that have redefined bacterial taxonomy. The most extensive studies regarding the phylogenetics of *Pseudomonas spp*. have been based on the 16S rRNA gene (Kersters et al., 1996; Anzai et al., 2000; Porteous et al.,

2002). The use of the 16S rRNA gene to infer phylogenetic relationships is criticized as it assumes one molecule reflects the evolutionary history of the organism, it is present in multiple copies and is subject to operon heterogeneity. Nelson et al. (2002) reports *P. putida* KT2440 to possess 7 rRNA operons. In addition, its slow rate of evolution limits the number of informative sites by which organisms are discriminated (Ait Tayeb et al., 2005). Protein-encoding genes are reported to evolve at a faster rate than rRNA encoding genes (Ochman & Wilson, 1987). Therefore, phylogenetic analysis using the *cpn*60 gene (encodes an essential 60kDa heat-shock chaperonin) is expected to provide greater resolution. The discriminatory ability of the *cpn*60 gene sequence to resolve closely related organisms is established and contains more phylogenetically informative data than 16S rRNA gene sequences (Marston et al. 1999; Brousseau et al., 2001). Like 16S rRNA, the *cpn*60 gene is an "informational" gene and thus less likely to be subject to lateral gene transfer. Unlike the 16S rRNA gene, there is usually only a single copy of the *cpn*60 gene in prokaryotes and it is not subject to operon heterogeneity (Hill et al., 2002). Overall this suggests that for a comprehensive phylogenetic analysis more than one target is required to overcome the disadvantages of analyses conducting using single gene targets.

Previously, Misko and Germida (2002) examined the taxonomic and functional diversity of pseudomonads associated with the roots of field-grown canola. That study demonstrated that among the rhizosphere bacteria the most commonly occurring genus was *Pseudomonas,* which accounted for ~35% (N=447) of the culturable root-associated population. The most commonly occurring species were *P. putida* (N=172) and *P. chlororaphis* (N=145), accounting for 38% and 32% of the root-associated *Pseudomonas* population, respectively. Collectively, these two species accounted for 70% of the pseudomonad population. In addition, sizeable populations of

P. syringae and *P. corrugata* were identified from the roots of field-grown canola. Characterization of their carbon substrate utilization profiles indicated that the pseudomonads were metabolically diverse with bacteria shown to be the same strain by FAME analysis to be metabolically different suggesting that functional redundancy exists among those pseudomonad isolates. The objectives of this study were: (i) to confirm the taxonomic identification and diversity of a subset of the pseudomonads previously isolated from the roots of field-grown canola using 16S rRNA and *cpn*60 gene sequencing and (ii) to compare the identifications obtained with the 16S rRNA and *cpn*60 genes to those obtained with FAME analysis.

3.2 Materials and Methods

3.2.1 *Pseudomonas* isolates

In 1998, bacteria were isolated from the rhizosphere and root interior of four cultivars of field-grown canola planted at a field site in Melfort, Saskatchewan (Misko & Germida, 2002). Three of the cultivars were transgenic (Exceed, Innovator, and Quest) and the other was non-transgenic (Excel). The bacteria were identified by their fatty acid methyl ester (FAME) profiles and the dominant genera composing 37% (N=447) of the identified culturable root-associated bacteria were pseudomonads (Misko & Germida, 2002). Of the 447 pseudomonads isolated from roots, 133 were randomly selected for further analysis in this study (rhizosphere, N=57, root interior, N=76).

3.2.2. PCR amplification and sequencing of the 16S rRNA gene

Amplifications of the 16S rRNA gene was conducted using whole cell lysates from pure cultures. Briefly, one colony from each pure culture was suspended in 100 μL of sterile distilled deionized water. To lyse the cells, suspensions were incubated for 10-12 min in a boiling water bath. Following lysis, the whole cell lysate was centrifuged at 14,000 rcf for 30-45 sec to

remove the bulk of the cellular debris. Amplification reactions were conducted in 25 μL volumes containing: 1 puRE Taq Ready-To-Go PCR bead (Amersham Biosciences Ltd.), 20 μL of sterile distilled deionized water, 2.5 μL of a 5 μM primer mix and 2.5 μL of the whole cell lysate supernatant.

All PCRs were performed in a Robocycler® Gradient 96 well Temperature Cycler (Stratagene) using thin-walled 0.2 mL reaction tubes. For each set of PCR reactions, a negative control was conducted containing all the PCR reagents excluding template DNA. The PCR cycle used to amplify the 16S rDNA region was: 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and a final extension time of 6 min at 72°C.

The primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 531R (5'-ACGCTTGCACCCTCCGTATT-3') were used to amplify the first 544 bp of the 16S rRNA gene (~1500bp). This primer system targets variable regions 1-3 of the bacterial 16S gene. Variable regions 1-3 exhibited less operon heterogeneity when pure cultures of pseudomonads were analyzed and thus were chosen for this study (Schmalenberger et al., 2001).
PCR amplification of the 16S rRNA gene segments resulted in products of single bands consisting of 544 bp. To visualize the PCR products, a 1% agarose gel made in 1x TBE [Tris(Tris(Hydroxymethyl)aminomethane)-boric acid-EDTA (ethylenediaminetetraacetic acid) buffer; in g l^{-1}: Tris, 10.8, EDTA, 0.93, boric acid, 5.5, pH 8.0] with 0.5 μg ml^{-1} ethidium bromide was used. Four μL were taken from each PCR reaction combined with 3 μL of 6x loading buffer (1 mL of 2.5% bromophenol blue, 1 mL of 2.5% xylene cyanol, 3 mL of glycerol, 5 mL of water) and 6 μL of sterile distilled deionized water and loaded into a well on the gel. For each gel, a low DNA mass ladder (Invitrogen) was used to determine the size and the

approximate concentration of the PCR product. The loaded gels were run in 1x TBE buffer for 1 h at 110 V. The bands were subsequently visualized by ultraviolet light.

The quantity of DNA was estimated by comparing band intensities with the low DNA mass ladder. A DNA quantity of ca.16 ng μL^{-1} was required to obtain a sequence from the product. PCR products of appropriate quantities without any contaminants were purified with the Wizard® PCR Preps DNA purification system (Promega) and sequenced.

DNA sequencing was conducted in collaboration with the Veterinary Infectious Disease Organization (VIDO), Saskatoon, Saskatchewan with a Beckman CEQ automatic DNA sequencing machine. Sequencing of the PCR products was performed using the fluorescence-based dideoxy termination method. Sequencing reactions were set up with the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman-Coulter), 0.5-1.0 µL of a 16 ng μL^{-1} purified PCR product and 1.6 µM sequencing primers. The sequencing primers were the same as the amplification primers. The conditions for the reactions were 20 sec at 96°C, 20 sec at 50°C, and 4 min at 60°C for 30 cycles. For sequencing of the 16S rRNA gene segment, all the isolates were sequenced in only one direction unless the sequence was of insufficient quality.

3.2.3 PCR amplification and sequencing of the *cpn*60 gene

Amplification and sequencing of the *cpn*60 gene from the *Pseudomonas* isolates was conducted in collaboration with Janet Hill at NRC-PBI (National Research Council Plant Biotechnology Institute) from cell pellets stored in a 10% glycerol solution. The primers H279 (5'-GAIIIIGCIGGIGAYGGIACIACIAC-3') and H280 (5'-YKIYKITCICCRAAICCIGGIGCYTT-3') were used to amplify the region of the *cpn*60 gene corresponding to nucleotides 274-828 of the *E. coli cpn*60 sequence (not including primer landing sites) (I=inosine, Y=C or T, R=G or A, K=G or T, S=G or C). This primer system

amplifies a 549-567 bp region of the *cpn*60 gene (~1640bp). The *cpn*60 gene encodes for a group I chaperonin (Cpn60) that is synonymous to GroEL and Hsp60. To produce PCR products from pure templates for direct sequencing, H729 (5'-CGCCAGGGTTTTCCCAGTCACGACGAIIIIGCIGGIGAYGGIACIACIAC-3') and H730 (5'-AGCGGATAACAATTTCACACAGGAYKIYKITCICCRAAICCIGGIGCYTT-3') (which contained the underlined standard M13 sequencing primer landing sites) were used.

3.2.2 Data analysis

Each 16S gene sequence was queried for similarities with BLAST (Altschul et al., 1990) and with the Ribosomal Database Project (RDP) Similarity Rank and Chimera Check programs (Maidak et al., 2000). Each *cpn*60 gene sequence was queried for similarities with the *cpn*60 database (Hill et al., 2004) and with BLAST (Altschul et al., 1990). Graphical correlations between 16S rRNA, *cpn*60 and FAME analyses were conducted in a similar manner as described by Ait Tayeb et al. (2005) with the exception that pairwise similarity values (psv) were analyzed. Graphical comparisons between the characterization methods were created using the psv for all possible isolate combinations. The computer programs PHYLLIP (Felsenstein, 1989) and MVSP 3.13 (Kovach Computing Services) were used to calculate the pairwise similarities between isolates using their 16S rRNA and *cpn*60 gene sequences, and their FAME profiles (Misko & Germida, 2002), respectively. Pairwise similarity values for each characterization method comparison were analyzed using correlation analysis (Pearson product moment coefficient) (Minitab v.14, Minitab Inc., State College, PA).

3.3 Results

3.3.1 Identification of bacteria isolated from the roots of field-grown canola by partial 16S rRNA and *cpn*60 gene sequencing

Partial 16S rRNA sequences were determined for 133 isolates (root interior, N=76, rhizosphere, N=57) comprising a subset of the pseudomonads previously identified by FAME analysis (Misko & Germida, 2002). Among the 133 rRNA sequences analyzed, none were found to be chimeric (data not shown). Similarity searches in the RDP and GenBank databases revealed that all the sequences belonged to the genus *Pseudomonas* (Table 3.1). In accordance with the study conducted by Misko and Germida (2002), the dominant species comprising 40% (N=53) of the examined pseudomonad population was *P. putida* (Table 3.2, Figure 3.1). Other commonly occurring species were *P. fluorescens*, *P. jessenii* and *P. migulae* comprising 20% (N=27), 17% (N=22), and 17% (N=22) of the examined pseudomonad population, respectively. However, none of the pseudomonad isolates was identified as belonging to the species *P. chlororaphis*.

Partial *cpn*60 gene sequences were determined for the same 133 pseudomonad isolates previously identified by both FAME and 16S rRNA analysis. Similarity searches in the *cpn*60 and GenBank databases revealed that all the sequences belong to the genus *Pseudomonas* (Table 3.1). In accordance with FAME and 16S rRNA analysis, the dominant species comprising 41% (N=55) of the examined pseudomonad population was *P. putida* (Table 3.2, Figure 3.1). Corresponding to the 16S rRNA analysis, *P. fluorescens* was the next most predominant species comprising 41% (N=54) of the examined root-associated pseudomonad population. The remaining 18% (N=24) of the examined pseudomonad *cpn*60 sequences were only able to be identified as belonging to a *Pseudomonas spp*.; greater resolution to the species level was not

Table 3.1. Identification of the *Pseudomonas spp.* isolated from the roots of field-grown canola as determined by FAME analysis, by 16S rDNA sequencing and by *cpn*60 gene sequencing.

Isolate code	FAME identification[a]	SIM index	16S rDNA identification	Percent Sequence Similarity	E value[b]	*cpn*60 identification	Percent Sequence Similarity	E value
1-1	*Pseudomonas chlororaphis*	0.620	*Pseudomonas putida*	99	0.0	*Pseudomonas fluorescens*	98	1.0E-93
1-3	*Pseudomonas putida*	0.469	*Pseudomonas putida*	99	0.0	*Pseudomonas fluorescens*	97	4.0E-94
1-4	*Pseudomonas chlororaphis*	0.426	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	97	4.0E-93
1-6	*Pseudomonas chlororaphis*	0.315	*Pseudomonas putida*	98	0.0	*Pseudomonas putida*	97	4.0E-93
1-7	*Pseudomonas putida*	0.653	*Pseudomonas fluorescens*	94	0.0	*Pseudomonas fluorescens*	97	4.0E-94
1-8	*Pseudomonas chlororaphis*	0.819	*Pseudomonas fluorescens*	95	5.0E-160	*Pseudomonas fluorescens*	98	2.0E-94
1-10	*Pseudomonas putida*	0.647	*Pseudomonas putida*	94	0.0	*Pseudomonas putida*	98	5.0E-94
1-11	*Pseudomonas putida*	0.668	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	5.0E-94
1-12	*Pseudomonas chlororaphis*	0.759	*Pseudomonas jessennii*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
1-14	*Pseudomonas chlororaphis*	0.811	*Pseudomonas putida*	94	3.0E-164	*Pseudomonas putida*	97	4.0E-93
1-15	*Pseudomonas chlororaphis*	0.437	*Pseudomonas putida*	98	0.0	*Pseudomonas putida*	97	4.0E-93
1-16	*Pseudomonas chlororaphis*	0.506	*Pseudomonas putida*	94	0.0	*Pseudomonas putida*	97	4.0E-93

[a] Isolates were identified using FAME analysis. Isolates with similarity indices (SIM) ≥0.3 were considered positively identified, whereas isolates with a SIM ≤0.3 were considered tentatively identified (Misko &Germida, 2002).

[b] Expect (E) value is the probability that an alignment score equal to the one found between a query and a database sequence would be found in comparisons between random sequences (Mount, 2001).

Table 3.1. continued.

Isolate code	FAME identification	SIM index	16S rDNA identification	Percent Sequence Similarity	E value	*cpn*60 identification	Percent Sequence Similarity	E value
1-17	*Pseudomonas putida*	0.881	*Pseudomonas tolaasii*	93	2.0E-126	*Pseudomonas fluorescens*	98	1.0E-93
1-18	*Pseudomonas putida*	0.801	*Pseudomonas jessenii*	99	0.0	*Pseudomonas putida*	97	4.0E-93
2-1	*Pseudomonas chlororaphis*	0.314	*Pseudomonas putida*	97	0.0	*Pseudomonas putida*	98	6.0E-94
2-2	*Pseudomonas putida*	0.675	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	5.0E-94
2-3	*Pseudomonas putida*	0.620	*Pseudomonas migulae*	98	0.0	*Pseudomonas sp.*	97	7.0E-94
2-4	*Pseudomonas putida*	0.529	*Pseudomonas migulae*	99	0.0	*Pseudomonas sp.*	97	7.0E-94
2-5	*Pseudomonas putida*	0.765	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	97	4.0E-93
2-6	*Pseudomonas chlororaphis*	0.389	*Pseudomonas putida*	98	0.0	*Pseudomonas fluorescens*	98	2.0E-94
2-8	*Pseudomonas putida*	0.583	*Pseudomonas fluorescens*	99	0.0	*Pseudomononas putida*	97	4.0E-93
2-9	*Pseudomonas chlororaphis*	0.915	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	5.0E-94
2-12	*Pseudomonas chlororaphis*	0.592	*Pseudomonas migulae*	99	0.0	*Pseudomonas sp.*	97	7.0E-94
2-14	*Pseudomonas putida*	0.541	*Pseudomonas migulae*	100	0.0	*Pseudomonas sp.*	97	2.0E-93
2-15	*Pseudomonas putida*	0.703	*Pseudomonas migulae*	98	0.0	*Pseudomonas putida*	97	4.0E-93
2-16	*Pseudomonas chlororaphis*	0.332	*Pseudomonas putida*	97	0.0	*Pseudomononas putida*	96	1.0E-92

Table 3.1. continued.

Isolate code	FAME identification	SIM index	16S rDNA identification	Percent Sequence Similarity	E value	*cpn*60 identification	Percent Sequence Similarity	E value
2-19	*Pseudomonas chlororaphis*	0.855	*Pseudomonas putida*	99	0.0	*Pseudomononas putida*	97	3.0E-92
2-20	*Pseudomonas putida*	0.928	*Pseudomonas jessenii*	99	0.0	*Pseudomonas putida*	98	5.0E-94
2-22	*Pseudomonas putida*	0.529	*Pseudomonas putida*	98	0.0	*Pseudomonas putida*	97	4.0E-93
4-4	*Pseudomonas chlororaphis*	0.437	*Pseudomonas putida*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
4-5	*Pseudomonas chlororaphis*	0.437	*Pseudomonas putida*	98	0.0	*Pseudomonas fluorescens*	97	4.0E-94
4-6	*Pseudomonas chlororaphis*	0.437	*Pseudomonas putida*	98	0.0	*Pseudomonas fluorescens*	97	1.0E-93
4-9	*Pseudomonas putida*	0.647	*Pseudomonas sp.*	98	0.0	*Pseudomonas putida*	98	5.0E-94
4-10	*Pseudomonas putida*	0.647	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	95	2.0E-90
4-11	*Pseudomonas putida*	0.647	*Pseudomonas putida*	97	0.0	*Pseudomonas putida*	98	5.0E-94
4-12	*Pseudomonas putida*	0.647	*Pseudomonas putida*	98	0.0	*Pseudomonas putida*	98	5.0E-94
4-13	*Pseudomonas chlororaphis*	0.759	*Pseudomonas jessennii*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
4-15	*Pseudomonas chlororaphis*	0.426	*Pseudomonas putida*	98	0.0	*Pseudomonas fluorescens*	97	4.0E-94
4-16	*Pseudomonas chlororaphis*	0.426	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	97	4.0E-93
4-19	*Pseudomonas chlororaphis*	0.315	*Pseudomonas putida*	99	0.0	*Pseudomononas putida*	97	4.0E-93

Table 3.1. continued.

Isolate code	FAME identification	SIM index	16S rDNA identification	Percent Sequence Similarity	E value	*cpn*60 identification	Percent Sequence Similarity	E value
4-20	*Pseudomonas chlororaphis*	0.315	*Pseudomonas putida*	97	0.0	*Pseudomonas putida*	97	4.0E-93
4-21	*Pseudomonas chlororaphis*	0.819	*Pseudomonas fluorescens*	98	0.0	*Pseudomonas fluorescens*	96	2.0E-91
4-23	*Pseudomonas putida*	0.653	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	97	4.0E-94
4-30	*Pseudomonas putida*	0.346	*Pseudomonas jessenii*	100	0.0	*Pseudomonas putida*	97	4.0E-93
4-31	*Pseudomonas putida*	0.346	*Pseudomonas jessenii*	99	0.0	*Pseudomonas putida*	97	4.0E-93
4-32	*Pseudomonas putida*	0.801	*Pseudomonas jessenii*	99	0.0	*Pseudomonas putida*	97	4.0E-93
4-33	*Pseudomonas putida*	0.801	*Pseudomonas jessenii*	99	0.0	*Pseudomonas putida*	97	4.0E-93
4-36	*Pseudomonas chlororaphis*	0.506	*Pseudomonas jessenii*	100	0.0	*Pseudomonas putida*	97	4.0E-93
4-44	*Pseudomonas putida*	0.667	*Pseudomonas putida*	100	0.0	*Pseudomonas putida*	98	6.0E-94
4-45	*Pseudomonas putida*	0.403	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	97	4.0E-94
4-46	*Pseudomonas putida*	0.853	*Pseudomonas fluorescens*	100	0.0	*Pseudomonas fluorescens*	97	4.0E-94
4-48	*Pseudomonas putida*	0.787	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	98	1.0E-94
4-51	*Pseudomonas putida*	0.619	*Pseudomonas stutzeri*	96	1.0E-92	*Pseudomonas fluorescens*	98	1.0E-94
4-54	*Pseudomonas putida*	0.872	*Pseudomonas putida*	96	4.0E-167	*Pseudomonas putida*	98	5.0E-94

Table 3.1. continued.

Isolate code	FAME identification	SIM index	16S rDNA identification	Percent Sequence Similarity	E value	*cpn*60 identification	Percent Sequence Similarity	E value
4-57	*Pseudomonas chlororaphis*	0.876	*Pseudomonas fluorescens*	93	3.0E-76	*Pseudomonas fluorescens*	98	6.0E-95
4-58	*Pseudomonas putida*	0.516	*Pseudomonas putida*	94	5.0E-112	*Pseudomononas putida*	97	2.0E-93
4-61	*Pseudomonas chlororaphis*	0.855	*Pseudomonas fluorescens*	96	4.0E-133	*Pseudomonas fluorescens*	97	4.0E-94
4-62	*Pseudomonas putida*	0.771	*Pseudomonas putida*	99	0.0	*Pseudomononas putida*	98	6.0E-94
4-63	*Pseudomonas chlororaphis*	0.676	*Pseudomonas fluorescens*	96	0.0	*Pseudomonas fluorescens*	98	2.0E-94
4-64	*Pseudomonas chlororaphis*	0.752	*Pseudomonas orientalis*	97	1.0E-157	*Pseudomonas fluorescens*	98	6.0E-94
4-65	*Pseudomonas putida*	0.618	*Pseudomonas fluorescens*	100	0.0	*Pseudomonas fluorescens*	98	1.0E-94
4-94	*Pseuduomonas chlororaphis*	0.323	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	5.0E-94
4-95	*Pseudomonas chlororaphis*	0.845	*Pseudomonas fluorescens*	98	0.0	*Pseudomonas fluorescens*	98	2.0E-94
4-96	*Pseudomonas chlororaphis*	0.850	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	98	2.0E-94
4-98	*Pseudomonas chlororaphis*	0.466	*Pseudomonas fluorescens*	98	2.0E-140	*Pseudomonas fluorescens*	97	1.0E-93
4-99	*Pseudomonas chlororaphis*	0.359	*Pseudomonas jessennii*	98	0.0	*Pseudomonas fluorescens*	97	2.0E-93
4-100	*Pseudomonas chlororaphis*	0.623	*Pseudomonas jessenii*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
4-101	*Pseudomonas chlororaphis*	0.406	*Pseudomonas jessennii*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93

Table 3.1. continued.

Isolate code	FAME identification	SIM index	16S rDNA identification	Percent Sequence Similarity	E value	*cpn*60 identification	Percent Sequence Similarity	E value
4-102	*Pseudomonas chlororaphis*	0.311	*Pseudomonas putida*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
4-103	*Pseudomonas putida*	0.370	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	94	4.0E-90
4-105	*Pseudomonas putida*	0.592	*Pseudomonas migulae*	99	0.0	*Pseudomonas sp.*	97	6.0E-94
4-106	*Pseudomonas chlororaphis*	0.458	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	5.0E-94
4-107	*Pseudomonas putida*	0.855	*Pseudomonas migulae*	99	0.0	*Pseudomonas fluorescens*	91	1.0E-87
4-108	*Pseudomonas putida*	0.832	*Pseudomonas migulae*	99	2.0E-162	*Pseudomonas sp.*	97	5.0E-93
4-109	*Pseudomonas putida*	0.709	*Pseudomonas migulae*	100	7.0E-168	*Pseudomonas sp.*	97	7.0E-94
4-110	*Pseudomonas putida*	0.547	*Pseudomonas migulae*	98	0.0	*Pseudomonas sp.*	97	5.0E-93
4-114	*Pseudomonas fluorescens*	0.877	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	97	4.0E-93
4-115	*Pseudomonas chlororaphis*	0.609	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	5.0E-94
4-139	*Pseudomonas chlororaphis*	0.311	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	97	4.0E-93
4-140	*Pseudomonas putida*	0.722	*Pseudomonas constantinii*	99	0.0	*Pseudomonas sp.*	97	6.0E-94
4-141	*Pseudomonas putida*	0.550	*Pseudomonas marginalis*	99	0.0	*Pseudomonas fluorescens*	99	3.0E-95
4-143	*Pseudomonas putida*	0.658	*Pseudomonas extremorientalis*	99	0.0	*Pseudomonas fluorescens*	98	6.0E-98

Table 3.1. continued.

Isolate code	FAME identification	SIM index	16S rDNA identification	Percent Sequence Similarity	E value	*cpn*60 identification	Percent Sequence Similarity	E value
4-151	*Pseudomonas putida*	0.779	*Pseudomonas fluorescens*	100	0.0.	*Pseudomonas fluorescens*	97	4.0E-94
4-152	*Pseudomonas putida*	0.456	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	97	4.0E-94
4-154	*Pseudomonas putida*	0.726	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	98	2.0E-94
4-156	*Pseudomonas putida*	0.597	*Pseudomonas migulae*	99	0.0	*Pseudomonas sp.*	97	7.0E-94
4-157	*Pseudomonas putida*	0.330	*Pseudomonas jessenii*	100	0.0	*Pseudomonas fluorescens*	97	2.0E-93
4-180	*Pseudomonas chlororaphis*	0.614	*Pseudomonas putida*	98	0.0	*Pseudomonas putida*	98	6.0E-94
4-183	*Pseudomonas chlororaphis*	0.837	*Pseudomonas jessennii*	99	0.0	*Pseudomonas putida*	98	6.0E-94
4-184	*Pseudomonas chlororaphis*	0.651	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	6.0E-94
4-185	*Pseudomonas chlororaphis*	0.318	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	6.0E-94
4-186	*Pseudomonas chlororaphis*	0.534	*Pseudomonas putida*	98	0.0	*Pseudomonas putida*	97	1.0E-93
4-192	*Pseudomonas chlororaphis*	0.628	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	5.0E-94
4-193	*Pseudomonas putida*	0.749	*Pseudomonas marginalis*	99	0.0	*Pseudomonas fluorescens*	98	6.0E-95
5-1	*Pseudomonas chlororaphis*	0.739	*Pseudomonas putida*	99	0.0	*Pseudomonas fluorescens*	97	4.0E-93
5-2	*Pseudomonas putida*	0.671	*Pseudomonas jessenii*	100	0.0	*Pseudomonas sp.*	97	5.0E-93

Table 3.1. continued.

Isolate code	FAME identification	SIM index	16S rDNA identification	Percent Sequence Similarity	E value	*cpn*60 identification	Percent Sequence Similarity	E value
5-3	*Pseudomonas chlororaphis*	0.547	*Pseudomonas migulae*	99	0.0	*Pseudomonas sp.*	97	7.0E-94
5-4	*Pseudomonas putida*	0.713	*Pseudomonas migulae*	98	0.0	*Pseudomonas sp.*	97	5.0E-93
5-5	*Pseudomonas putida*	0.520	*Pseudomonas migulae*	98	0.0	*Pseudomonas sp.*	97	7.0E-94
5-6	*Pseuduomonas chlororaphis*	0.797	*Pseudomonas jessenii*	100	0.0	*Pseudomonas putida*	97	4.0E-93
5-7	*Pseudomonas fluorescens*	0.487	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	97	4.0E-93
5-9	*Pseudomonas fluorescens*	0.478	*Pseudomonas jessenii*	99	0.0	*Pseudomonas putida*	97	4.0E-93
5-10	*Pseudomonas putida*	0.735	*Pseudomonas migulae*	99	0.0	*Pseudomonas sp.*	97	6.0E-94
5-15	*Pseudomonas putida*	0.362	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas sp.*	97	5.0E-93
5-17	*Pseudomonas corrugata*	0.790	*Pseudomonas migulae*	99	0.0	*Pseudomonas sp.*	97	7.0E-94
5-18	*Pseudomonas corrugata*	0.800	*Pseudomonas migulae*	99	0.0	*Pseudomonas sp.*	97	5.0E-93
5-19	*Pseudomonas putida*	0.324	*Pseudomonas putida*	100	4.0E-161	*Pseudomonas putida*	98	5.0E-94
5-22	*Pseudomonas chlororaphis*	0.398	*Pseudomonas fluorescens*	96	3.0E-118	*Pseudomonas fluorescens*	97	2.0E-93
5-25	*Pseudomonas putida*	0.853	*Pseudomonas putida*	96	2.0E-98	*Pseudomonas fluorescens*	98	7.0E-95
5-27	*Pseudomonas chlororaphis*	0.372	*Pseudomonas putida*	100	0.0	*Pseudomononas putida*	96	1.0E-92

Table 3.1. continued.

Isolate code	FAME identification	SIM index	16S rDNA identification	Percent Sequence Similarity	E value	*cpn*60 identification	Percent Sequence Similarity	E value
5-28	*Pseudomonas chlororaphis*	0.351	*Pseudomonas jessenii*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
5-30	*Pseudomonas chlororaphis*	0.754	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	98	2.0E-94
5-31	*Pseudomonas chlororaphis*	0.873	*Pseudomonas fluroescens*	94	2.0E-104	*Pseudomonas fluorescens*	97	4.0E-94
5-36	*Pseudomonas chlororaphis*	0.786	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	97	4.0E-94
5-37	*Pseudomonas putida*	0.520	*Pseudomonas migulae*	98	0.0	*Pseudomonas sp.*	97	7.0E-94
5-39	*Pseudomonas chlororaphis*	0.334	*Pseudomonas jessenii*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
5-40	*Pseudomonas chlororaphis*	0.528	*Pseudomonas jessenii*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
5-41	*Pseudomonas chlororaphis*	0.759	*Pseudomonas putida*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
5-42	*Pseudomonas chlororaphis*	0.504	*Pseudomonas putida*	99	0.0	*Pseudomonas putida*	98	6.0E-94
5-44	*Pseudomonas syringae*	0.770	*Pseudomonas migulae*	98	0.0	*Pseudomonas sp.*	97	5.0E-93
5-45	*Pseudomonas syringae*	0.517	*Pseudomonas migulae*	98	0.0	*Pseudomonas sp.*	97	6.0E-94
5-46	*Pseudomonas chlororaphis*	0.693	*Pseudomonas putida*	99	0.0	*Pseudomonas fluorescens*	97	2.0E-93
5-108	*Pseudomonas syringae*	0.901	*Pseudomonas constantinii*	98	0.0	*Pseudomonas sp.*	97	3.0E-93
5-109	*Pseudomonas chlororaphis*	0.837	*Pseudomonas jessenii*	99	0.0	*Pseudomonas putida*	98	6.0E-94

Table 3.1. continued.

Isolate code	FAME identification	SIM index	16S rDNA identification	Percent Sequence Similarity	E value	*cpn*60 identification	Percent Sequence Similarity	E value
5-110	*Pseudomonas putida*	0.658	*Pseudomonas fluorescens*	100	0.0	*Pseudomonas fluorescens*	99	3.0E-95
5-111	*Pseudomonas chlororaphis*	0.820	*Pseudomonas jessennii*	98	0.0	*Pseudomonas putida*	98	6.0E-94
5-112	*Pseudomonas corrugata*	0.747	*Pseudomonas putida*	98	0.0	*Pseudomonas putida*	97	2.0E-93
5-113	*Pseudomonas corrugata*	0.827	*Pseudomonas migulae*	99	0.0	*Pseudomonas sp.*	97	6.0E-94
5-115	*Pseudomonas putida*	0.896	*Pseudomonas putida*	99	0.0	*Pseudomonas fluorescens*	97	3.0E-93
5-117	*Pseudomonas chlororaphis*	0.548	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	98	2.0E-94
5-118	*Pseudomonas putida*	0.831	*Pseudomonas putida*	98	0.0	*Pseudomonas putida*	98	5.0E-94
5-119	*Pseudomonas savastanoi pv. oleae*	0.819	*Pseudomonas migulae*	97	0.0	*Pseudomonas sp.*	97	7.0E-94
5-120	*Pseudomonas chlororaphis*	0.875	*Pseudomonas fluorescens*	99	0.0	*Pseudomonas fluorescens*	99	3.0E-95

Table 3.2. *Pseudomonas* isolates within canola cultivars and root zones as determined by FAME analysis, by 16S rDNA sequencing and by *cpn*60 gene sequencing. E = root interior, R = rhizosphere.

Pseudomonas spp.	Number of isolates identified by FAME analysis[a]								Total	Number of isolates identified by 16S rRNA sequencing[b]								Total	Number of isolates identified by *cpn*60 gene sequencing[c]								Total
	Plant cultivar									Plant cultivar									Plant cultivar								
	Exceed		Excel		Innovator		Quest			Exceed		Excel		Innovator		Quest			Exceed		Excel		Innovator		Quest		
	E	R	E	R	E	R	E	R		E	R	E	R	E	R	E	R		E	R	E	R	E	R	E	R	
chlororaphis	15	8	3	15	5	2	5	10	63	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	0
constantinii	-	-	-	-	-	-	-	-	0	1	1	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	0
corrugata	-	2	-	-	-	2	-	-	4	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	0
extremeorientalis	-	-	-	-	-	-	-	-	0	-	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	0
fluorescens	-	1	-	-	-	2	-	-	3	-	3	2	-	-	2	12	8	27	7	6	4	10	3	1	13	10	54
jessenii	-	-	-	-	-	-	-	-	0	-	3	-	7	10	1	-	1	22	-	-	-	-	-	-	-	-	0
marginalis	-	-	-	-	-	-	-	-	0	-	1	-	-	1	-	-	-	2	-	-	-	-	-	-	-	-	0
migulae	-	-	-	-	-	-	-	-	0	2	3	7	2	5	3	-	-	22	-	-	-	-	-	-	-	-	0
orientalis	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	0
putida	8	5	14		15	3	11	3	59	19	6	7	8	4	3	2	4	53	14	8	7	5	11	4	3	3	55
savastanoi	-	1	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	0
stutzeri	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	0
syringae	-	1	-	2	-	-	-	-	3	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	0
tolaasii	-	-	-	-	-	-	-	-	0	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	0
Other spp.	-	-	-	-	-	-	-	-	0	-	-	1	-	-	-	-	-	1	2	4	6	2	6	4	-	-	24
	41		34		29		29		133	41		34		29		29		133	41		34		29		29		133

[a] Bacteria were isolated from the roots of canola plants grown at a field site in Melfort, SK in 1998. Isolates were identified using fatty acid methyl ester analysis. Isolates with similarity indices (SIM) of 0.3 or greater were considered positively identified, whereas isolates with a SIM of less than 0.3 were considered tentatively identified (Misko and Germida, 2002).

[b] Isolates were identified using partial 16S rDNA sequencing. Isolates with an E value of 0.0 and a percent sequence similarity ≥ 97.5 were considered positively identified, whereas isolates with an E value > 0.0 and/or a percent sequence similarity < 97.5 were considered tentatively identified.

[c] Isolates were identified using partial cpn60 gene sequencing. Isolates with an E value of 0.0 and a percent sequence similarity ≥ 97.5 were considered positively identified, whereas isolates with an E value > 0.0 and/or a percent sequence similarity < 97.5 were considered tentatively identified.

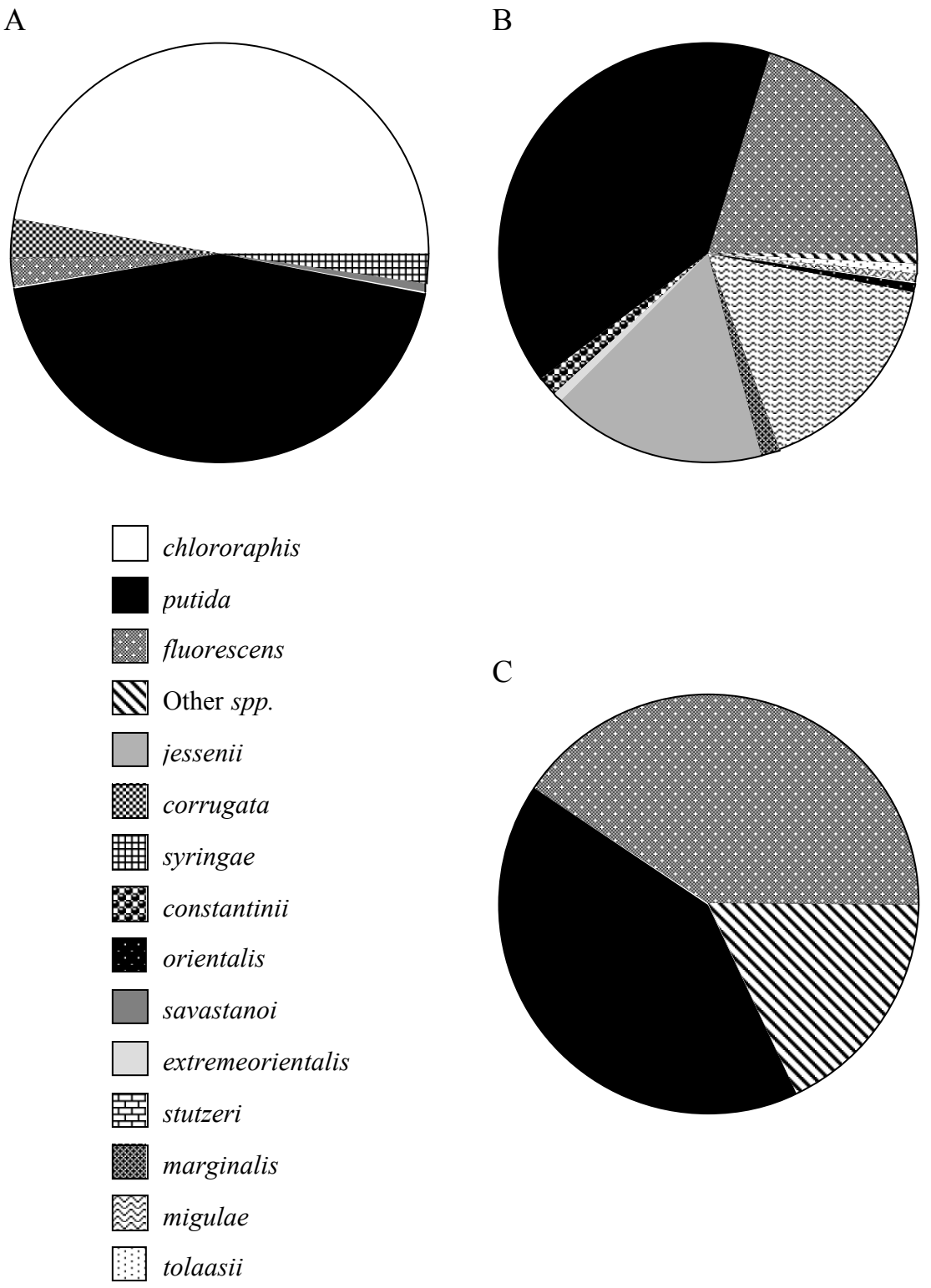

Figure 3.1. Speciation of the *Pseudomonas* isolates as determined by A) FAME analysis, B) 16S rDNA sequencing and C) *cpn*60 gene sequencing. Proportions were calculated as the percentages of each species as determined by the identification method.

possible. As was determined by the 16S rRNA sequence analysis, none of the pseudomonad isolates was identified as belonging to the species *P. chlororaphis.*

3.3.2 Comparison of FAME identifications with identifications conducted by 16S rRNA and *cpn*60 gene sequencing

Comparison of the FAME identifications to the *cpn*60 and the 16S rRNA identifications of the 133 pseudomonad isolates indicated that all methods agreed on the genus identification, but at a species level the FAME identification agreed with the 16S rRNA identification only 13% (N=17) of the time and with the *cpn*60 identification only 17% (N=23) (Table 3.1). Species identifications determined from all three analyses agreed for 11% (N=14) of the pseudomonad isolates. Pseudomonad isolates previously identified by FAME analysis as *P. chlororaphis* (N=63) were re-identified by 16S rRNA sequencing as *P. putida* (N=33), *P. fluorescens* (N=14), *P. jessenii* (N=13) and various other *Pseudomonas* (N=3). Of the 63 pseudomonad isolates previously identified by FAME analysis as *P.chlororaphis,* 33 were re-identified by *cpn*60 gene sequencing as *P. fluorescens*, 28 as *P. putida* and 2 as various other *Pseudomonas spp*.. The absence of *P. chlororaphis* species from the 16S rRNA and *cpn*60 identifications corresponded to 47% of the discrepancy between the FAME and 16S rRNA identifications, and between the FAME and *cpn*60 identifications. No other major trend was observed in the re-identification of the isolates by partial gene sequencing.

For each combination of types, 16S rRNA/FAME (Figure 3.2a), *cpn*60/FAME (Figure 3.2b) and *cpn*60/16S rRNA (Figure 3.2c) the level of correlation was determined by comparing the pairwise similarity values (psv) for all possible combination of isolates. In the *cpn*60/16S rRNA comparison, the majority of points are located between $psv_{16S\ rRNA}$ of 0.72 to 1.0 (represents similarities of 72 to 100%) and psv_{cpn60} of 0.86 to 1.0 (represents similarities of 89 to 100%), with a second group of fewer points located between $psv_{16S\ rRNA}$ of 0.37 to 0.47

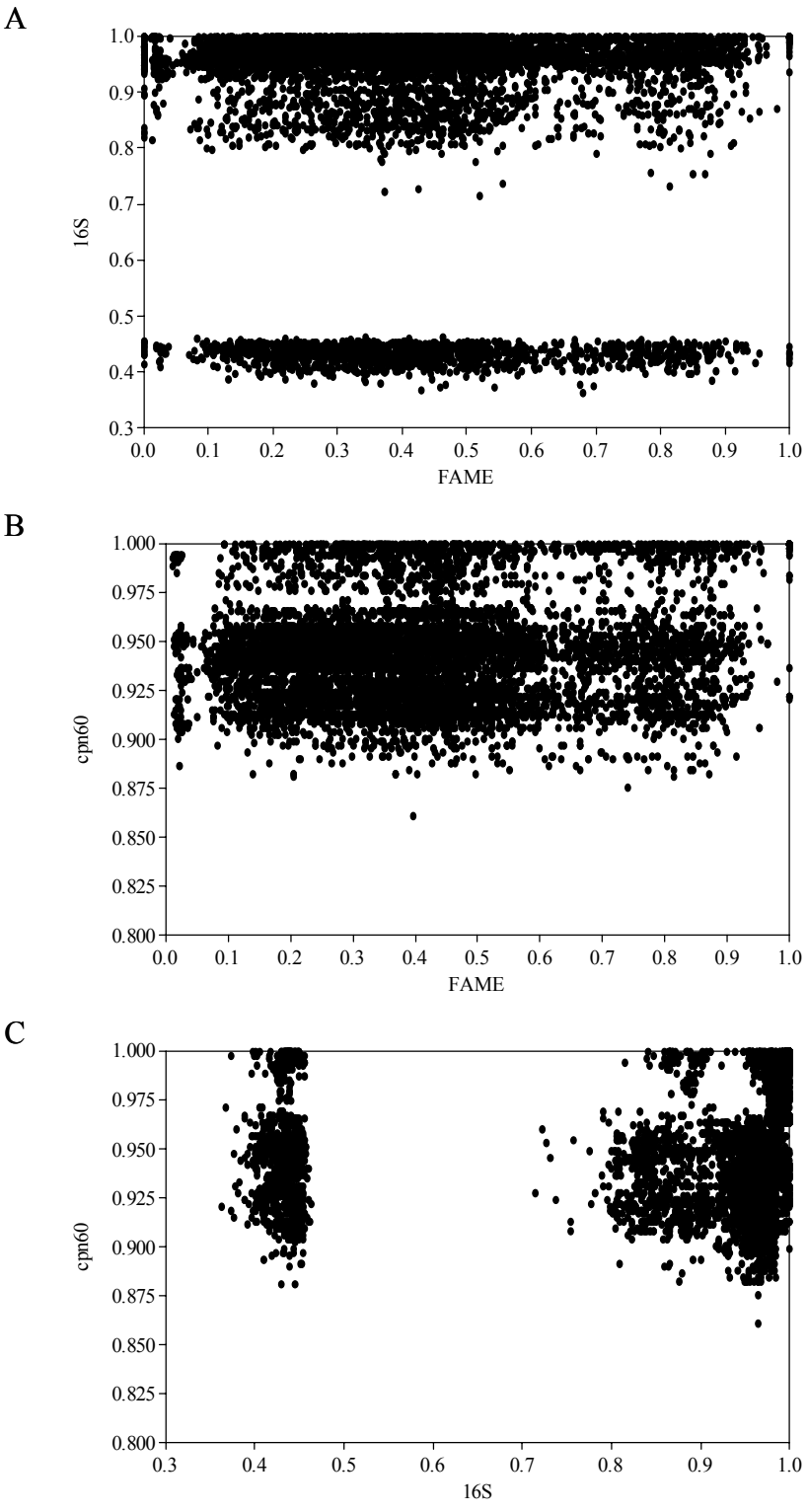

Figure 3.2. Graph showing the correlation between pairwise similarities of (A) the 16S rRNA gene and FAME profiles, (B) the *cpn*60 gene and FAME profiles and (C) the 16S rRNA and the *cpn*60 genes.

(represents similarities of 37 to 47%) and psv_{cpn60} of 0.86 to 1.0 (represents similarities of 89 to 100%). The majority of the pseudomonad isolates exhibited high levels of both 16S rRNA and *cpn*60 gene sequence similarity as is evident from their psv. The second cluster of points represents isolates with high *cpn*60 psv, but relatively low 16S rRNA psv. The second cluster of isolates is constituted by *Pseudomonas spp.* The high *cpn*60 psv limits the taxonomic resolving power of the *cpn*60 gene for the *Pseudomonas spp*. The majority of 16S rRNA psv were also high (0.72 to 1.0) which indicates a relationship between the 16S rRNA and *cpn*60 psv (i.e. isolates highly related by both sequences analyzed) (Correlation coefficient = 0.241, P = 0.000). The clustering of high psv_{cpn60} with relatively low $psv_{16S\ rRNA}$ indicates that they are more closely related by their *cpn*60 gene sequences than by their 16S rRNA sequences which could be the result of either greater operon heterogeneity in their 16S rRNA genes or a lack of correlation between the two genes in those isolates (i.e. isolates are polyphyletic).

The $psv_{16S\ rRNA}$ and the psv_{cpn60} were high in comparison to the psv_{FAME} which ranged from 0.0 to 1.0. However, correlation analysis of the 16S rRNA/FAME and the *cpn*60/FAME relationships indicates a weak but significant correlation with coefficients of 0.031 (P = 0.003) and 0.042 (P = 0.000), respectively.

3.4 Discussion

Pseudomonads, particularly the fluorescent pseudomonads, are common rhizosphere bacteria accounting for a significant portion of the culturable rhizosphere bacteria. Their ability to utilize a wide range of compounds for carbon and energy makes them well suited to the rhizosphere. The predominance of pseudomonads in the rhizosphere has been reported for several plants, including wheat, canola, ryegrass, bentgrass and clover (Grayston et al., 1998; Siciliano and Germida, 1999). Some *Pseudomonas spp*. improve plant growth and plant health

and are implicated in the natural suppressiveness of certain soils to various soilborne diseases (Weller, 1988; Lemanceau, 1992;) whereas others are involved in the biodegradation of natural or man-made toxic chemical compounds (Holloway, 1992). The presence and diversity of *Pseudomonas spp*. in the rhizosphere is important because of their ability to influence plant and soil health.

Many different *Pseudomonas* species have been identified, but the diversity and predominance of different *Pseudomonas* species in rhizosphere soils is just beginning to be explored. Members of the genus *Pseudomonas* are an exceptionally diverse group of organisms exhibiting high levels of physiological and genetic diversity even within species (Bossis et al., 2000). In the current study, the taxonomic diversity of a subset of pseudomonads isolated from the roots of field-grown canola was examined using 16S rRNA and *cpn*60 gene sequencing. A subset of 133 isolates (root interior, N=76, rhizosphere, N=57) was randomly selected from the 447 pseudomonads previously identified by FAME analysis (Misko and Germida, 2002). Based on the 16S rRNA and *cpn*60 gene sequences, all the isolates belong to the genus *Pseudomonas* (Table 3.1). This study affirmed the genus identifications as determined by Misko and Germida (2002) and as a result confirms their finding that members of the genus *Pseudomonas* were predominant in the canola rhizosphere as determined by culture-dependent methods. Siciliano et al. (1999) studied the taxonomic diversity of culturable bacterial populations associated with the roots of field-grown canola. They found that pseudomonads were the most numerically abundant bacteria, accounting for ca. 12% of the root-associated bacterial community. Similar findings have also been reported for sugar beet (Rainey et al., 1994), flax (Lemanceau et al., 1995) and tomato plants (Lemanceau et al., 1995). The results of this study affirm that the canola root isolates are members of the genus *Pseudomonas*.

Taxonomy of the pseudomonads using 16S rRNA sequences showed the dominant species comprising 40% (N=53) of the examined pseudomonad population as *P. putida* (Table 3.2, Figure 3.1). Other commonly occurring species were *P. fluorescens*, *P. jessenii* and *P. migulae* comprising 20% (N=27), 17% (N=22), and 17% (N=22) of the examined pseudomonad population, respectively. Similar results were obtained using the *cpn*60 gene sequences with *P. putida* comprising 41% (N=55) of the examined pseudomonad population (Tables 3.1 & 3.2, Figure 3.1). *P. fluorescens* was the next most predominant species comprising 41% (N=54) of the examined root-associated pseudomonad population. The remaining 18% (N=24) of the examined pseudomonad *cpn*60 sequences were only able to identify the sequences as belonging to a *Pseudomonas* sp., but unable to provide greater resolution to the species level. Analysis of the gene sequences did not identify any of the pseudomonad isolates as belonging to the specie *P. chlororaphis.*

The speciation of the rhizosphere pseudomonad subset exhibited similarities to the findings of Misko and Germida (2002). They found the dominant species to be *P. putida* accounting for 38% of the root-associated *Pseudomonas* population, followed by *P. chlororaphis* which accounted for 32%. Collectively, these two species accounted for 70% of the culturable pseudomonad population. In addition, they identified sizeable populations of *P. syringae* and *P. corrugata* from the roots of field-grown canola. Lottman and Berg (2001) examined the pseudomonads commonly found in association with the roots of potato plants. They found the predominant species to be *P. putida*, *P. fluorescens*, *P. chlororaphis* and *P. syringae*. Similar results have been reported for tomato and flax plants (Latour et al., 1996; Clays-Josserand et al., 1999). These studies found that *P. putida*, *P. fluorescens* and *P. chlororaphis* were the most numerically abundant species. In my study, the most predominant members of the

pseudomonads were *P. putida* and *P. fluorescens* (Figure 3.1). My findings in conjunction with the literature suggest that within the genus *Pseudomonas* the fluorescent pseudomonads are the predominant colonizers of the roots of a variety of plants as determined by culture-dependent methodologies.

The ability of fluorescent pseudomonads to dominate the rhizosphere is explained by the many properties of these bacteria that enable them to be competitive colonizers. For example, the common phenotypic trait that is shared among the fluorescent pseudomonads is the production of fluorescent, iron-binding siderophores, such as pyoverdine, that are produced when cells are cultured on low iron media (Meyer, 2000; Kwon et al., 2005). In addition to siderophores, some fluorescent pseudomonads also produce antibiotics, and plant growth regulators, and are able to degrade or transform various pesticides and soil organic pollutants (Kwon et al., 1995)

Comparison of FAME, 16S rRNA gene and *cpn*60 gene typing of the isolates indicated that all methods agreed 100% on the genus identification, but at the species level the FAME identification agreed with the 16S identification only 13% of the time and with *cpn*60 identification only 17% of the time. Species identifications determined from all three analyses agreed for 11% of the pseudomonad isolates. Pseudomonad isolates previously identified by FAME analysis as *P. chlororaphis* (N=63) were re-identified by 16S rRNA sequencing as *P. putida* (N=33), *P. fluorescens* (N=14), *P. jessenii* (N=13) and various other *Pseudomonas*. (N=3). Of the 63 pseudomonad isolates previously identified by FAME analysis as *P.chlororaphis,* 33 have been re-identified by *cpn*60 gene sequencing as *P. fluorescens*, 28 as *P. putida* and 2 as various other *Pseudomonas spp*.. The absence of *P. chlororaphis* species from

the 16S rRNA and *cpn*60 identifications accounted for 47% of the discrepancy between the FAME and 16S rRNA identifications, and between the FAME and *cpn*60 identifications.

Discrepancies in species identifications between FAME and the gene typing methodologies could result from problems with the FAME database (e.g. insufficient representation of *Pseudomonas* species or failure to update the FAME profiles within the database) and/or FAME being a phenotypic classification method. Phenotype analysis does not always reflect the genotype of the strain, especially if the strain has faced severe nutrient conditions, which have led to phenotypic differentiation (Sazakli et al., 2005). Furthermore, the taxonomic discrepancies could be a result of the operational taxonomic units (OTU) used to define a species by the MIDI FAME-GC system (Barnett et al., 1999). Since all isolates were examined in the same stage of growth and under controlled conditions to minimize variation, the influence of phenotypic plasticity (i.e. bacteria readily produce new phenotypes in the laboratory) and/or phenotypic conversion was minimized (Barnett et al., 1999). Several studies have documented the large heterogeneity among the fluorescent *Pseudomonas spp*. especially *P. fluorescens*, *P. putida* and *P. syringae* that does not permit the resolution of species within the complexes by phenotypic or phylogenetic assessment (Grimont et al., 1996; Dawson et al., 2002; Ait Tayeb et al., 2005). The absence of *P. chlororaphis* from the identifications conducted using gene sequences can also be explained by the limited resolution of most identification methods. As Misko and Germida (2002) report, *P. chlororaphis* isolates can be discriminated from *P. putida* and *P. fluorescens* by FAME analysis, whereas Dawson et al. (2002) found that the use of DNA based typing methods resulted in the clustering of *P. chlororaphis* isolates with either *P. fluorescens* or *P. putida* isolates. In this study, two partial gene sequences (16S rRNA and *cpn*60) were analyzed as opposed to full-length gene sequences. The utilization of full-length

gene sequences could have provided greater resolution between the species in this study, but reports in the literature suggest that despite the utilization of full-length gene sequences resolution of fluorescent pseudomonads to the species level is difficult (Bossis et al., 2000; Dawson et al., 2002). This instability among *Pseudomonas* species results from the disagreement between phenotypic and genotypic OTUs. For example, a phenotypic OTU does not necessarily correspond to a genotypic OTU and vice versa. This study, in agreement with the literature, documents the high degree of heterogeneity among fluorescent *Pseudomonas spp*. and the need for further taxonomic investigation.

For each combination of types, 16S rRNA/FAME, *cpn*60/FAME and *cpn*60/16S rRNA the level of correlation was determined by pairwise similarities between isolates (Figure 3.2). Comparison of the FAME analysis with the 16S rRNA and *cpn*60 genes indicated a weak correlation between the levels of similarity. However, a relation was identified in which isolates appeared to present a high level of similarity of their 16S rRNA (0.715 to 1.000) together with a high range of similarity of their *cpn*60 gene (0.861 to 1) (correlation coefficient = 0.241, P = 0.000).

Analysis of the pairwise similarities between isolates based on comparison of the *cpn*60 and 16S genes suggested that the evolution rates of the two genes were similar for each isolate and accurately represent the evolution of the isolate. Given their high correlation, it would be expected that comparison of each sequence from the same isolate would lead to the same identification, but this is not the case and reaffirms the need for further taxonomic investigation into *Pseudomonas spp*. Furthermore, the resolution between the isolates is slightly greater using the 16S rRNA sequence data as indicated by a wider range of pairwise similarities compared to those obtained using the *cpn*60 pairwise similarities. This finding contrasts with the findings of

Marston et al. (1999) and Brousseau et al. (2001) that conclude the *cpn*60 gene sequences of *Bartonella spp*. and *Streptococcus spp*. provide greater taxonomic resolution than the corresponding 16S rRNA gene sequences. However, the degree of homogeneity within genera varies considerably and conclusions made for one genus may not correlate with other. Several studies have reported high levels of heterogeneity within the genus *Pseudomonas* (Dawson et al., 2002; Meyer, 2002; Ait-Tayeb et al., 2005).

Weak correlation was found between the similarity indices of the genes and the FAME profiles. The lack of congruence could be the result of divergent evolution rates between functional and operational or "essential" genes (Ochman and Wilson, 1987), the presence of MGEs, inaccuracies in the identification method or the lack of correlation between phenetic and genetic based OTU. The results of our study confirmed the genus identifications of the canola root isolates as determined by Misko and Germida (2002) and also the need for further taxonomic investigation into the speciation of fluorescent pseudomonads.

4.0 PHENOTYPIC AND PHYLOGENETIC CHARACTERIZATION OF SELECTED ROOT-ASSOCIATED PSEUDOMONADS.

4.1 Introduction

The concept of phenotypic variability describes the degree to which an organism's phenotype is determined by its genotype. Typically, bacteria are classified using a polyphasic approach encompassing both phenetic and phylogenetic classifications. The majority of phylogenetic studies are conducted utilizing one or more chromosomal genetic determinants and thus indicate chromosomal evolutionary distance. However, phenetic classifications do not always reflect phylogenetic relatedness (Urbach et al., 1997). Phenetic classifications represent the evolution of the entire bacterial genome in the sense of showing the end products of evolution. Unlike phylogenetic classifications, phenetic classifications are influenced by MGEs. This could result in bacteria that are phenotypically "plastic" in that a single genotype may produce many different phenotypes. Phenotypic variability results almost exclusively through mutation and the acquisition of genes by horizontal transfer systems, e.g. plasmids, bacteriophages, transposons or integrons (Bailey et al., 2001). Several MGEs are associated with *Pseudomonas spp*. ranging from self-transmissible plasmids encoding xenobiotic degradation genes to transposons carrying multiple antibiotic resistance. Pseudomonads are Gram-negative flagellated rod-shaped bacteria that can form associations with the roots of several plants. Among the rhizobacteria, the genus *Pseudomonas* is of particular importance due to its wide distribution, and its ability to use a wide range of compounds as carbon and energy sources. *Pseudomonas spp.* have been observed to play vital roles in nutrient cycling, to exhibit plant

growth promoting behaviour, and to inhibit several phytopathogens by acting as biological control agents.

I examined the phenotypic variation within a subset of pseudomonads (N=133) isolated from the rhizosphere and root-interior of four cultivars of field-grown canola (Melfort, SK). The pseudomonad isolates were characterized according to their 16S rRNA and *cpn*60 gene sequences and selected phenotypic properties (FAME profiles, antibiotic resistance, extracellular enzyme production, and carbon substrate utilization).

4.2 Materials and Methods

4.2.1 *Pseudomonas* isolates

The same *Pseudomonas* isolates were used as described in Section 3.2.1.

4.2.2 Phenotypic characterization and numerical taxonomy

To characterize the phenotypes of the isolated pseudomonads, 104 different phenotypic properties were assessed by Misko & Germida (2002) which included extracellular enzyme production (N = 9) and carbon substrate utilization (N = 95). To supplement their phenotypic data, the antibiotic resistance of the pseudomonads was assessed to six different antibiotics; tetracycline (200 $\mu g\ ml^{-1}$), gentamycin (200 $\mu g\ ml^{-1}$), ampicillin (500 $\mu g\ ml^{-1}$), chloramphenicol (200 $\mu g\ ml^{-1}$), streptomycin (200 $\mu g\ ml^{-1}$) and nalidixic acid (200 $\mu g\ ml^{-1}$) and the data subsequently reanalyzed. Antibiotic resistance was indicated by the presence of growth following a 1 week incubation at 28°C on 1/10 Tryptic Soy Agar (Difco) containing the appropriate antibiotic. The phenotypic data were converted into a binary matrix that was analyzed using the simple matching similarity coefficient. The simple matching similarity coefficient matrix was subjected to UPGMA analysis and a phenogram was created using average linkage procedure. The calculation of simple matching similarity coefficients and the construction of the UPGMA dendograms were conducted using the Multivariate Statistics

Package v.3.1. *Pseudomonas* phenotypes were also compared by principal component analysis (PCA) using the correlation matrix (Minitab v.12, Minitab Inc., State College, PA). The principal component data were subsequently analyzed using analysis of variance (ANOVA).

4.2.3 PCR amplification and sequencing of the 16S rRNA gene

Refer to section 3.2.2.

4.2.4 PCR amplification and sequencing of the *cpn*60 gene

Refer to section 3.2.3.

4.2.5 Sequence and phylogenetic analyses.

The nucleotide sequences were aligned using the CLUSTAL X program (Thompson et al., 1994) applying default parameters. Each alignment was visualized and edited using GeneDoc (Nicholas et al., 1997) to conform to structural information. Phylogenetic trees were constructed using programs from the PHYLIP package (Felsenstein, 1989) including SEQBOOT (100 replicates), DNAPARS, DNADIST (Jukes-Cantor model), NEIGHBOR (neighbor-joining model) and CONSENSE. Phylogenetic trees were visualized using Tree Explorer.

4.3 Results

4.3.1 Phenotypic characterization and relatedness of pseudomonads isolated from the roots of field-grown canola

The phenotypic relatedness of the pseudomonad isolates was determined through PCA and through cluster analysis based on FAME profiles (Misko & Germida, 2002) and phenotypic characterizations (i.e. antibiotic resistance, extracellular enzyme production and carbon substrate utilization). The final phenotypic data set contained phenotypic information for 133 isolates and 110 unit characters. The pooled variance over 110 phenotypic assays per isolate was 0.075 or 7.5%. All of the isolates from a single cultivar or root zone were grouped together for analysis.

It was determined that the isolates from the Excel, Exceed and Innovator canola varieties tended to exhibit similar phenotypic properties, but that slight phenotypic variation existed between isolates from the Quest variety and the remaining cultivars (Figure 4.1A). PCA analysis of the fatty acid composition of the bacteria confirmed the trend determined by their phenotypic properties in that isolates from the Excel and Exceed canola varieties exhibited similar profiles, but no variation existed between isolates from the Quest variety and the remaining cultivars (Figure 4.2A). Significant variation existed between the examined phenotypes (i.e. antibiotic resistance, extracellular enzyme production and carbon substrate utilization) of isolates from different root zones suggesting that isolates from the rhizosphere had different phenotypic properties than the isolates from the root interior (Figure 4.1B). Analysis of the fatty acid composition of the pseudomonads according to root zones indicated that there was no significant variation in FAME compositions (Figure 4.2B). This suggested that the phenotypes of pseudomonads were similar between cultivars, but not between isolates from different root zones, but that FAME profiles were similar in isolates regardless of cultivar or root zone. Substrates that had a large influence on principal components (PCs) 1 and 2 are indicated in Table 4.1.

Table 4.1. Loading values for phenotypic properties having the most influence on principal components 1 and 2.

PC 1		PC 2	
Phenotype	PC score	Phenotype	PC score
N-Acetyl-D-glucosamine	-0.191	Melibiose	0.198
Gentiobiose	-0.199	Bromosuccinic acid	-0.219
Melibiose	-0.191	Succinamic acid	-0.251
Maltose	-0.188	L-Threonine	-0.202
β-Methyl-D-glucoside	-0.187	Glucose-1-phosphate	0.209
D-Raffinose	-0.191	Glucose-6-phosphate	0.202

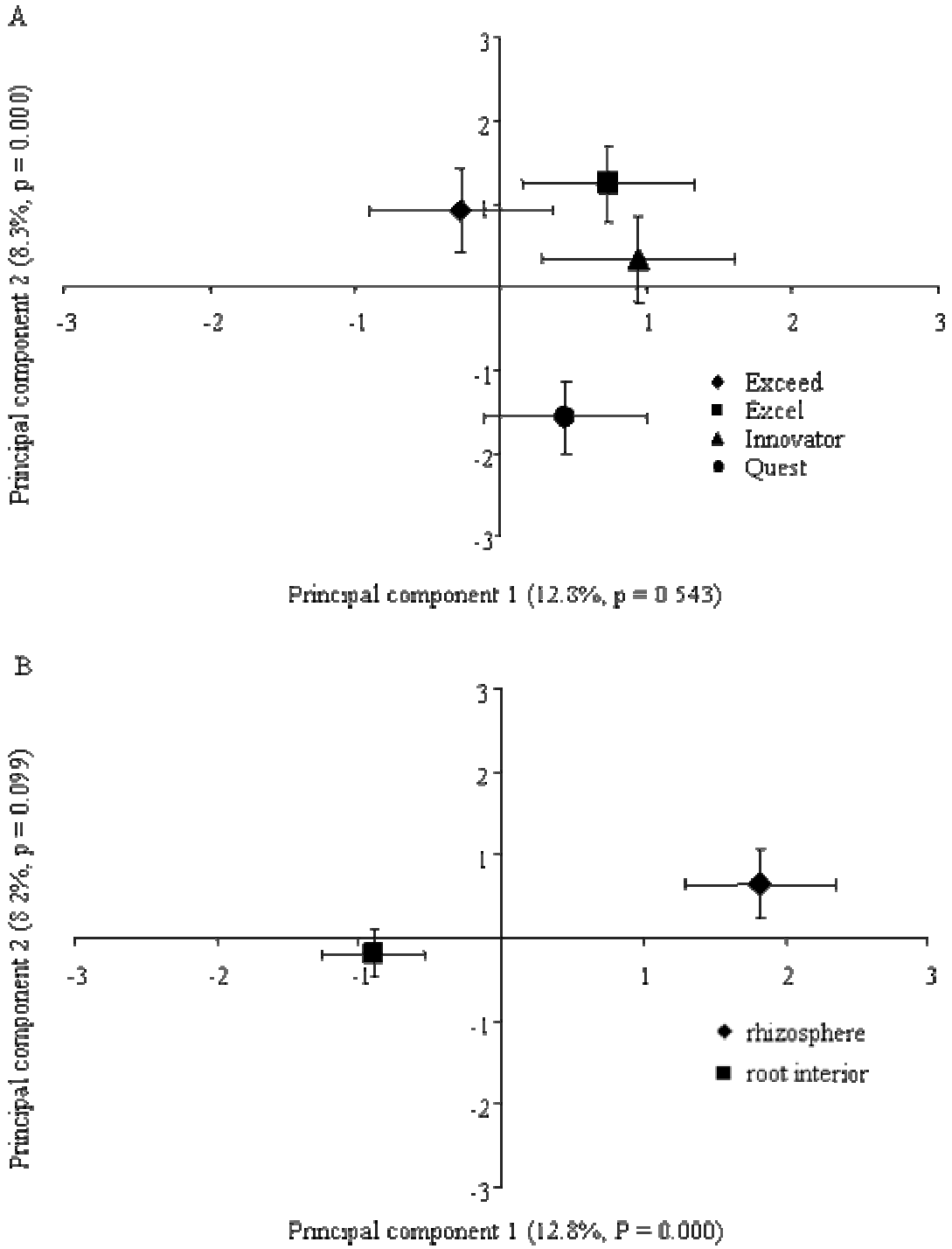

Figure 4.1. Principal component analysis of the phenotypic variation among pseudomonads isolated from the roots of four field-grown canola cultivars as determined by phenotypic profiles according to A) cultivar and to B) root zone. Points are the average for all isolates from each cultivar or root zone, plus standard error. Percentages indicate the proportion of the variation between groups of isolates that are accounted for by PC1 and PC2. PCs marked with a $p \leq 0.005$ value indicate a significant effect as determined by ANOVA.

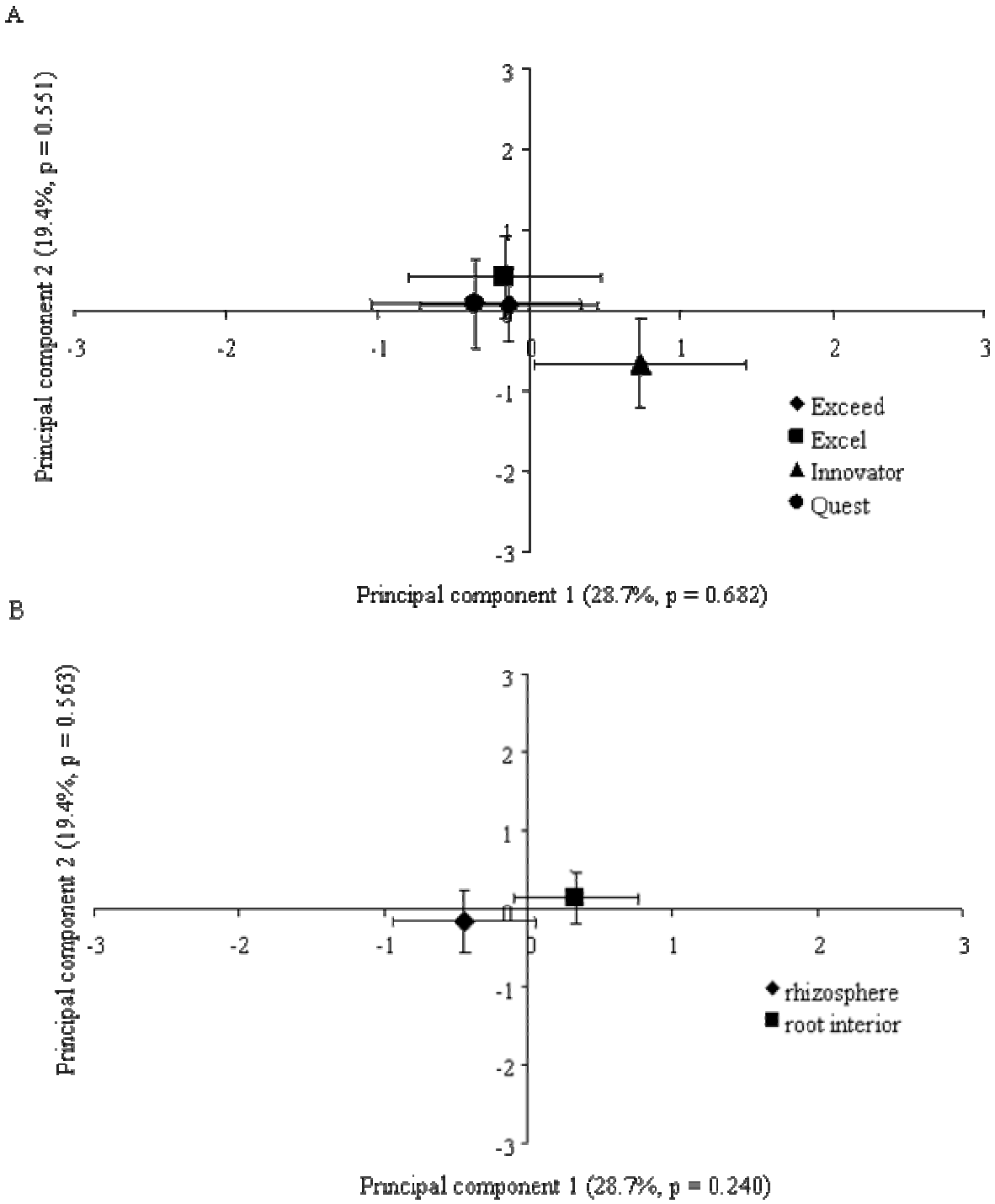

Figure 4.2. PCA of the phenotypic variation among pseudomonads isolated from the roots of four field-grown canola cultivars as determined by FAME profiles according to A) cultivar and to B) root zone. Points are the average for all isolates from each cultivar or root zone, plus standard error. Percentages indicate the proportion of variation between groups of isolates that are accounted for by PC1 and PC2. PCs marked with a $p \leq 0.005$ value indicate a significant effect as determined by ANOVA.

4.3.2 Phylogenetic characterization and relatedness of pseudomonads isolated from the roots of field-grown canola

On the basis of 16S rRNA and *cpn*60 gene sequence data, the majority of the pseudomonad isolates were highly related phylogenetically and exhibited greater than 93% sequence similarity between isolates. Two major clusters were observed namely the *Pseudomonas fluorescens* complex and the *P. putida* complex. Phylogenetic analysis suggested that the phylogeny of root associated pseudomonads had no effect on their associations with different root zones (Figures 4.3, 4.4, 4.5 & 4.6) or cultivars (Figures 4.3, 4.4, 4.7 & 4.8). To ensure reproducibility, the 16S rRNA region of 4 isolates was re-sequenced 3 times. The average variance over three sequences for an isolate was 5% or 27 bases.

4.3.3 Phenotypic and phylogenetic variability within a subset of root-associated pseudomonads

Cluster analysis of the phenotypic properties of the subset of 133 rhizosphere pseudomonads exhibited little correlation with their phylogenetic relationships. In the majority of situations, the isolates that grouped into a phylogenetic cluster had less than 75-80% similarity among their phenotypic traits despite a close evolutionary relationship as determined by 16S rRNA and *cpn*60 gene sequencing (Figures 4.9-4.11). For example, pseudomonad isolates 4-101 and 4-13 that are phylogenetically identical exhibit little correlation among their phenotypic properties (~76% phenotypic similarity). This trend is observed for several other pairwise comparisons between isolates such as 5-28 and 5-41, 4-99 and 1-14, and 4-94 and 4-192. The opposite situation whereby pseudomonad isolates that are phylogenetically more distant exhibit high levels of phenotypic similarity is plausible, but the close phylogenetic relationships of the pseudomonad isolates within the examined population do not permit this observation.

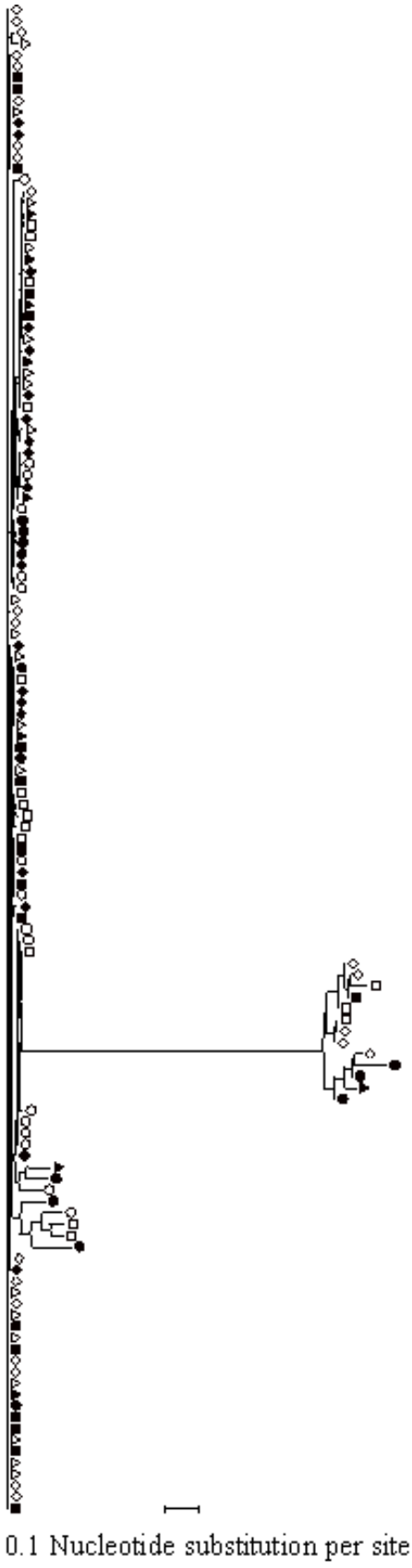

Figure 4.3. Phylogenetic comparison between *Pseudomonas spp*. isolated from the root interior (open symbols) and rhizosphere (closed symbols) of field-grown canola cultivars, Exceed (diamonds), Excel (squares), Innovator (triangles) and Quest (circles) as determined by analysis of the 16S rRNA gene.

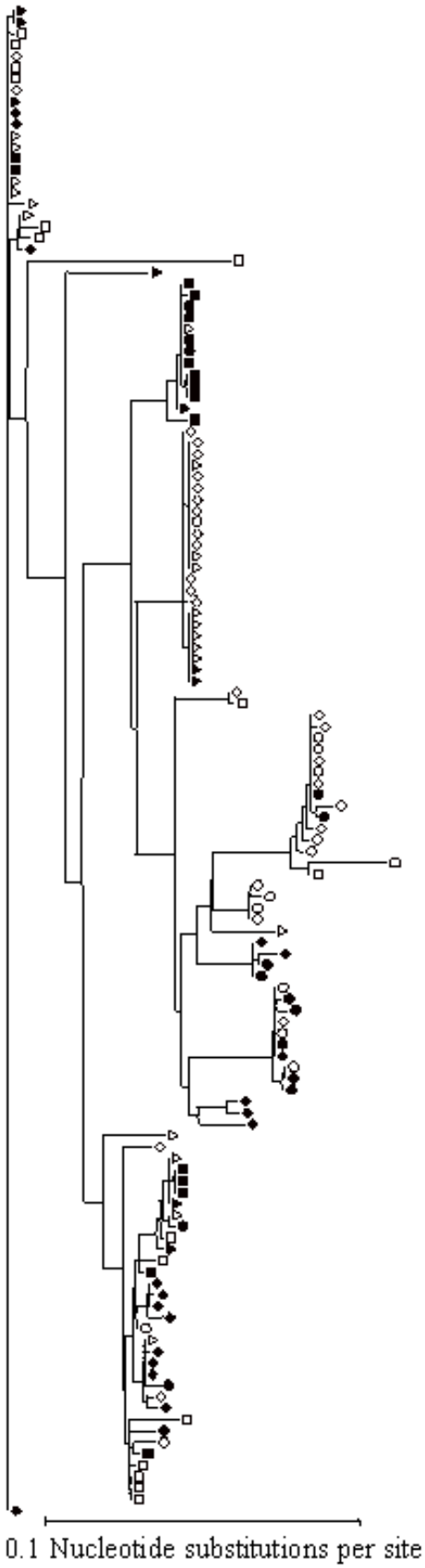

Figure 4.4. Phylogenetic comparison between *Pseudomonas spp*. isolated from the root interior (open symbols) and rhizosphere (closed symbols) of field-grown canola cultivars, Exceed (diamonds), Excel (squares), Innovator (triangles) and Quest (circles) as determined by analysis of the *cpn*60gene.

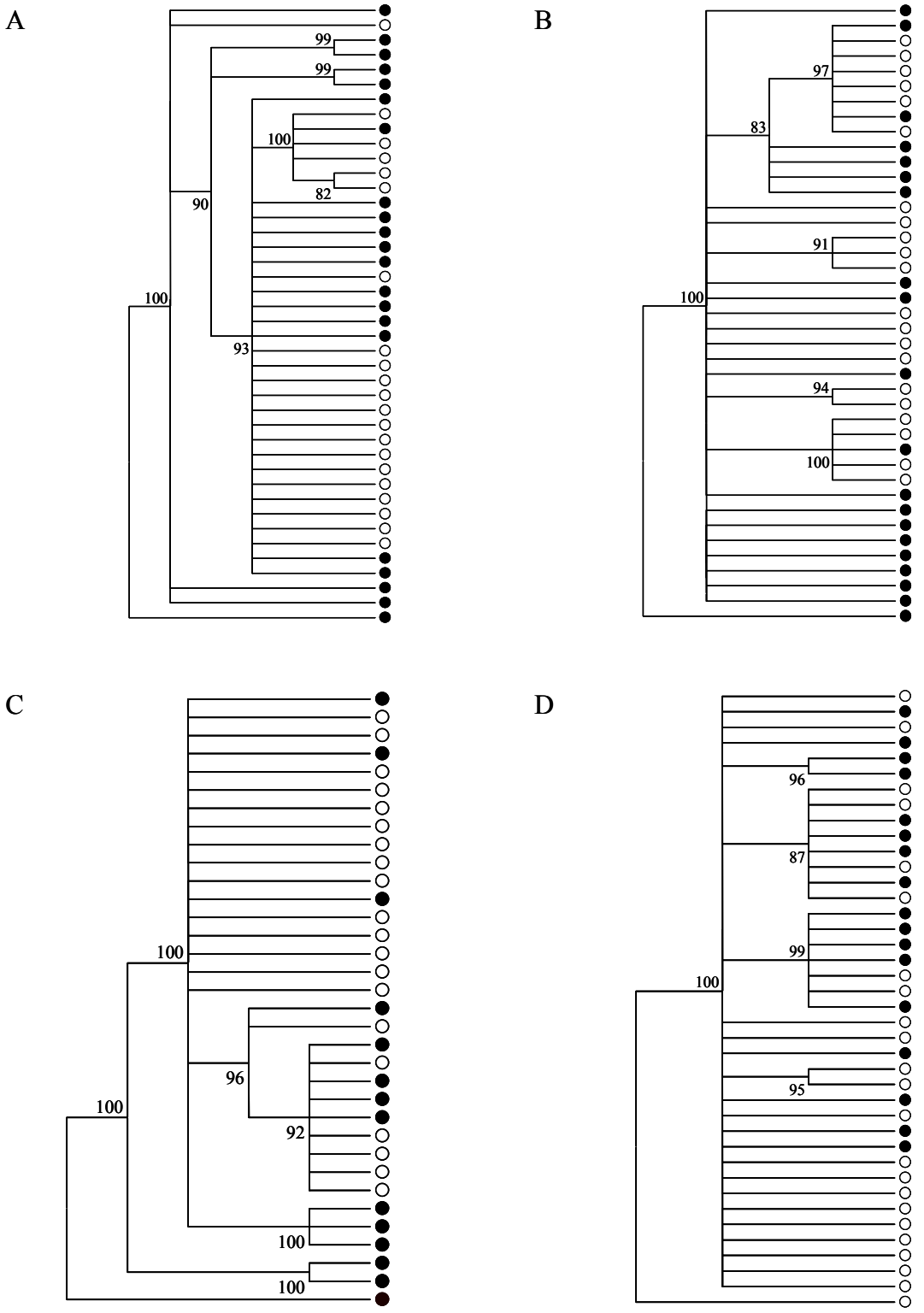

Figure 4.5. Phylogenetic comparison between *Pseudomonas spp.* isolated from the root interior (open circles) and rhizosphere (closed circles) of field-grown canola cultivars A) Exceed, B) Excel, C) Innovator and D) Quest based on analysis of the 16S rRNA gene. Nodes with bootstrap values of less than 80 are included within one cluster and are not phylogenetically different (Anzai et al., 2001). Branch length does not represent evolutionary distance between isolates.

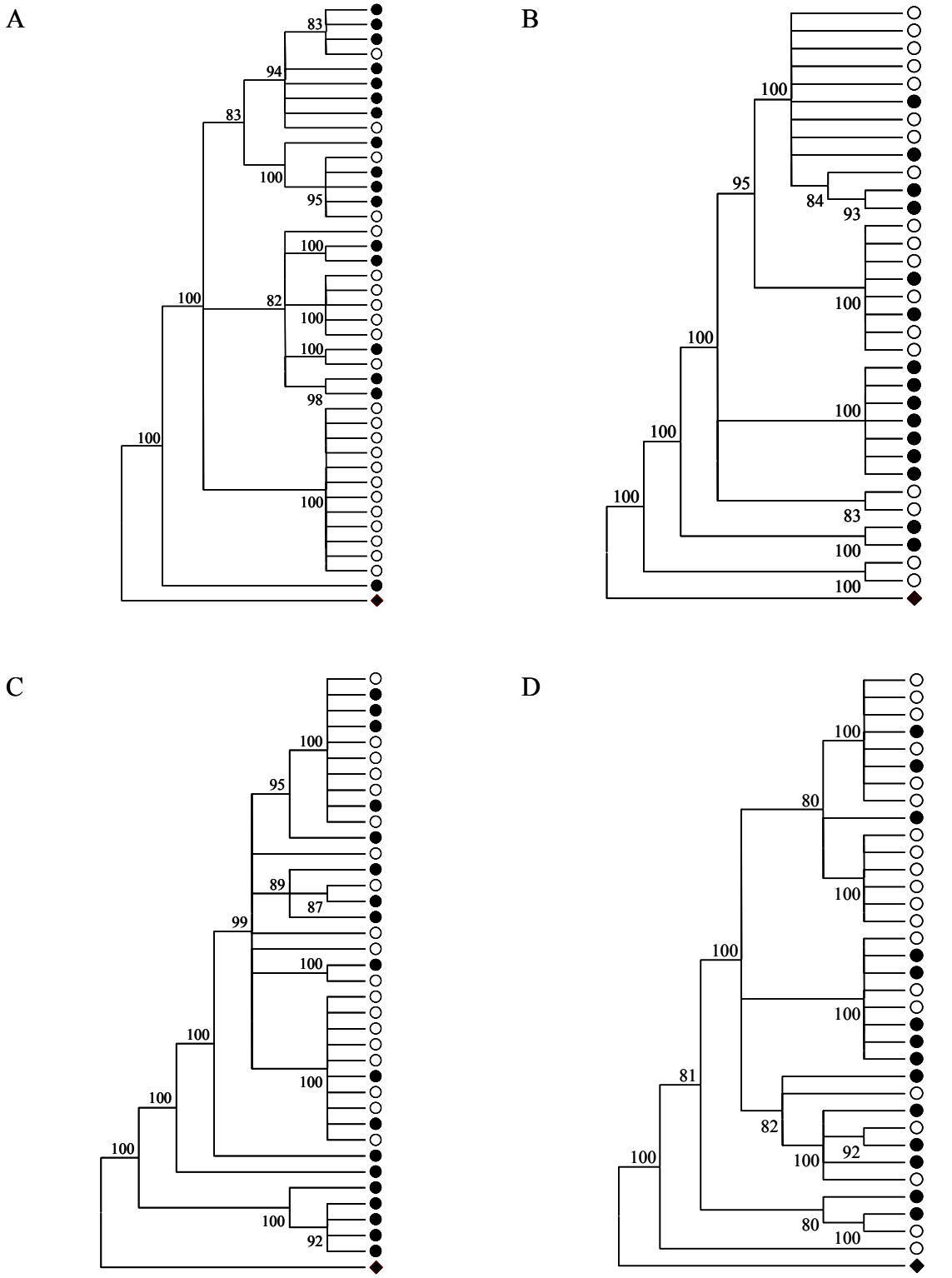

Figure 4.6. Phylogenetic comparison between *Pseudomonas spp*. isolated from the root interior (open circles) and rhizosphere (closed circles) of field-grown canola cultivars A) Exceed, B) Excel, C) Innovator and D) Quest based on analysis of the *cpn*60 gene. Closed diamond is the outgroup sequence from *E.coli* K12. Nodes with bootstrap values of less than 80 are included within one cluster and are not phylogenetically different (Anzai et al., 2001). Branch length does not represent evolutionary distance between isolates.

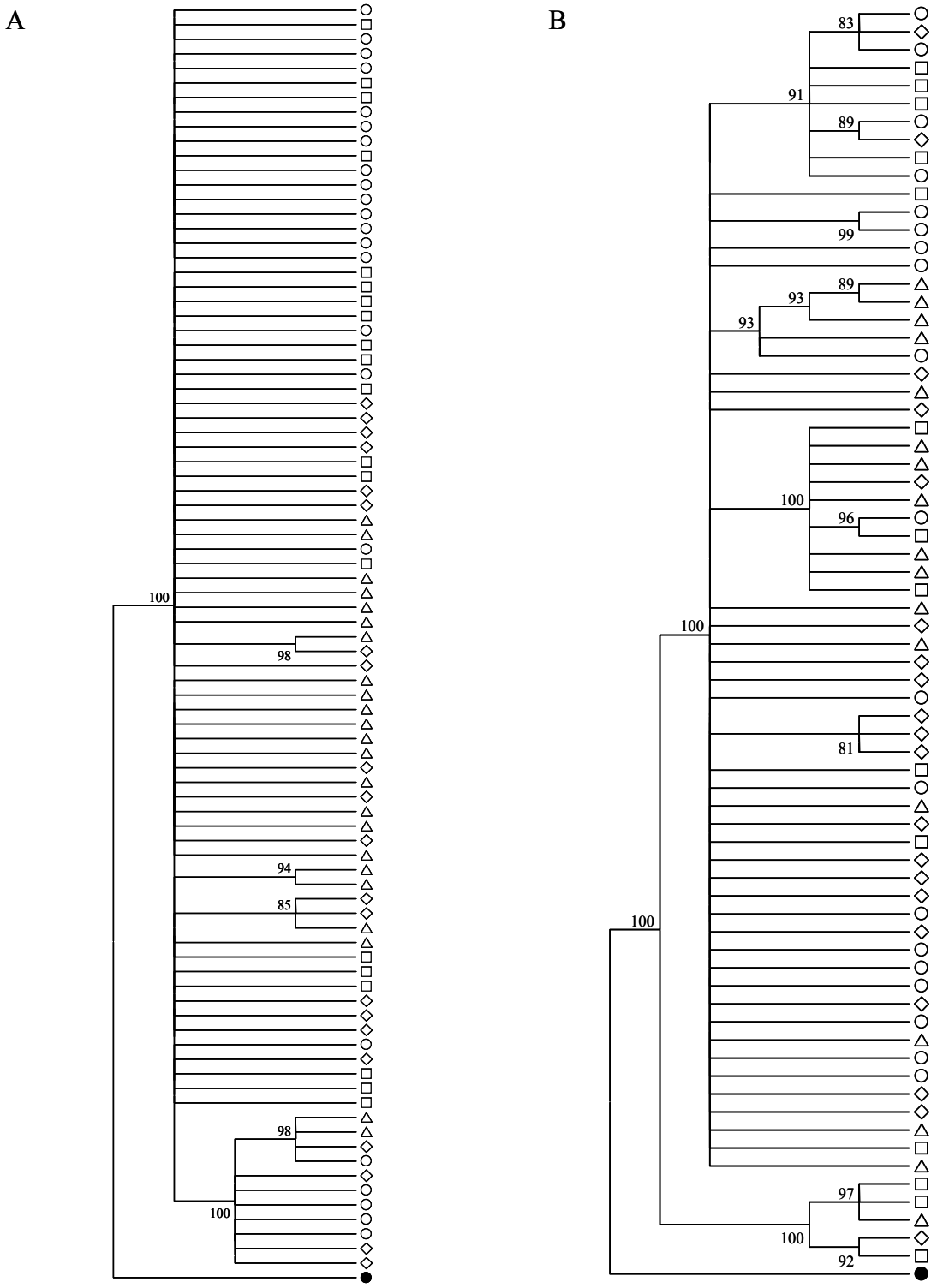

Figure 4.7. Phylogenetic comparison between pseudomonads isolated from the A) root interior and B) rhizosphere of four field-grown canola cultivars based on analysis of the 16S rRNA gene. Nodes with bootstrap values of less than 80 are included within one cluster and are not phylogenetically different (Anzai et al., 2001). Branch length does not represent evolutionary distance between isolates. (closed circle – *E.coli* K12, open circles – Exceed, open diamonds – Excel, open squares, Innovator, open triangles – Quest).

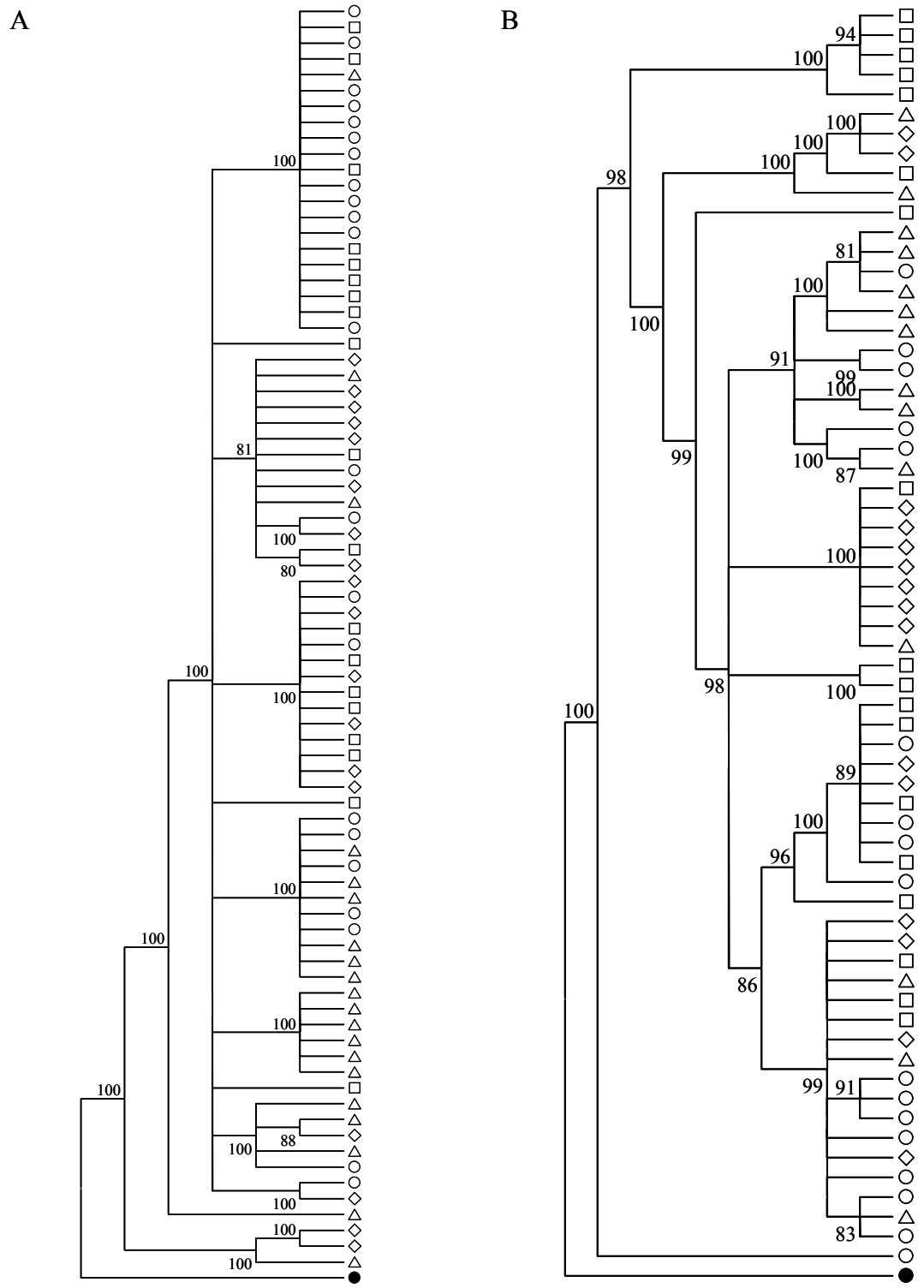

Figure 4.8. Phylogenetic comparison between *Pseudomonas spp*. isolated from the A) root interior and B) rhizosphere of four field-grown canola cultivars based on analysis of the *cpn*60 gene. Nodes with bootstrap values of less than 80 are included within one cluster and are not phylogenetically different (Anzai et al., 2001). Branch length does not represent evolutionary distance between isolates. (closed circle = *E.coli* K12, open circles = Exceed, open diamonds = Excel, open squares = Innovator, open triangles = Quest).

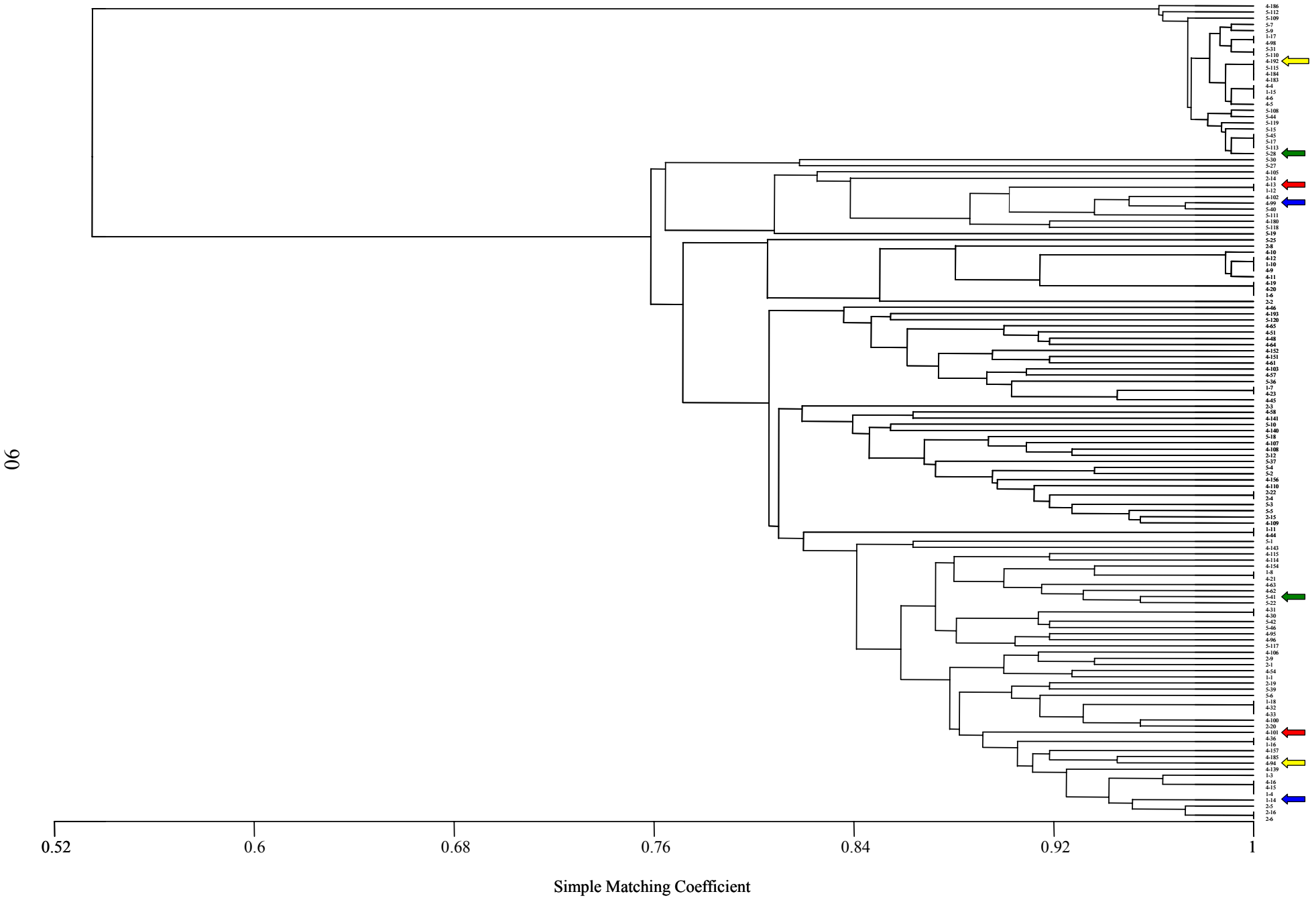

Figure 4-9. Phenotypic comparison between pseudomonads isolated from the roots of field-grown canola based on 110 selected phenotypic unit characters. For comparison purposes, yellow arrows represent 4-94 and 4-192, red arrows represent 4-101 and 4-13, blue arrows represent 4-99 and 1-14, and green arrows represent 5-28 and 5-41.

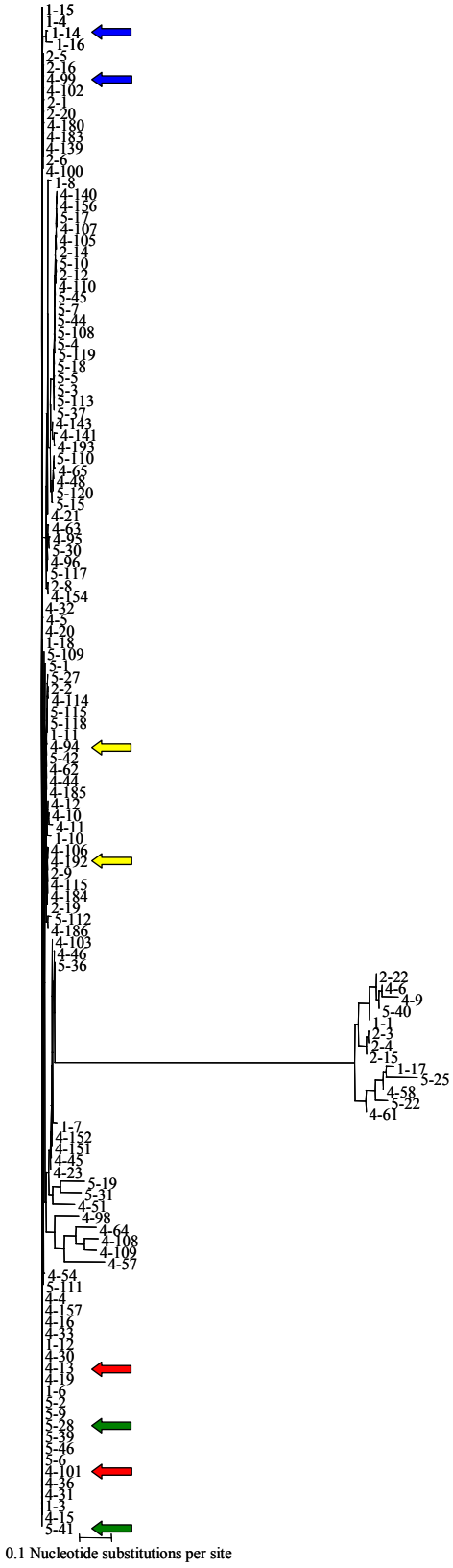

Figure 4.10. Phylogenetic comparison between pseudomonads isolated from the roots of field-grown canola based on analysis of the 16S rRNA gene. For comparison purposes, yellow arrows represent 4-94 and 4-192, red arrows represent 4-101 and 4-13, blue arrows represent 4-99 and 1-14, and green arrows represent 5-28 and 5-41.

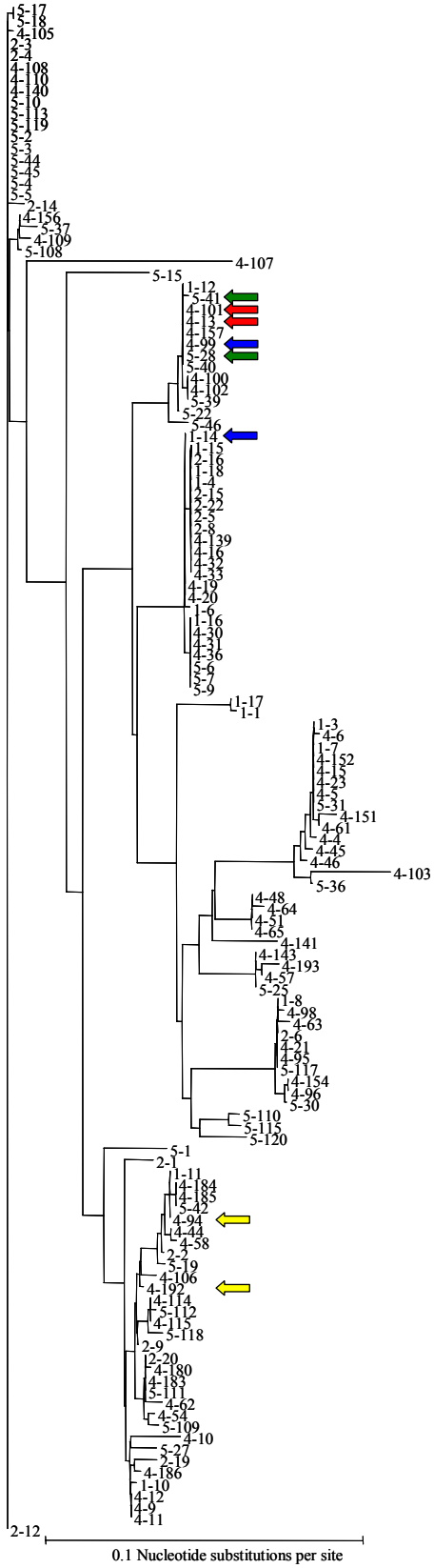

Figure 4.11. Phylogenetic comparison between pseudomonads isolated from the roots of field-grown canola based on analysis of the *cpn*60 gene. For comparison purposes, yellow arrows represent 4-94 and 4-192, red arrows represent 4-101 and 4-13, blue arrows represent 4-99 and 1-14, and green arrows represent 5-28 and 5-41.

4.4 Discussion

Due to the ubiquity and versatility of pseudomonads, there is considerable interest in exploiting these bacteria for a diverse range of biotechnological applications, such as the improvement of fossil fuels by desulphurization (Galan et al., 2000), the remediation of contaminated sites (Dejonghe et al., 2000) and as agents of plant growth promotion and plant pest control (Walsh et al., 2001). Despite the extensive examination of *Pseudomonas*, there is a lack of knowledge concerning the degree and source of *Pseudomonas spp*. diversity. The members of the genus *Pseudomonas* are an exceptionally diverse group of organisms exhibiting high levels of physiological and genetic diversity even within species. Their ability to colonize a wide range of habitats suggests a remarkable degree of physiological and genetic diversity. Several papers document microbial diversity at the community and at the organism level, but few examine the causes or ecological significance of this diversity. Diversity arises and is maintained through the interaction between ecological and genetic factors. The ultimate cause of diversification is mutation, but the variation generated by both mutation and recombination (including lateral gene transfer) is sorted and shaped by selective pressure and genetic drift (Spiers et al., 2000). Genetic factors are the ultimate determinants of patterns of diversity for without genetic variation there can be no evolution or divergence. Mutation and selection alone are powerful evolutionary forces, but nevertheless the ubiquitous nature of *Pseudomonas spp.* leads many to wonder if they are inherently more evolvable or if lateral gene transfer is largely the cause of their diversity. In this study, the phenotypic and phylogenetic variation within a subset of pseudomonads (N=133) isolated from the rhizosphere and root-interior of four cultivars of field-grown canola was examined.

In accordance with the FAME analysis conducted by Misko & Germida (2002), phylogenetic analysis of the partial 16S rRNA and *cpn*60 gene sequences suggested that the examined subset of pseudomonads found in the different root zones and different canola cultivars was homogeneous

(Figures 4.3-4.8). Several studies have reported significant differences between bacterial communities associated with different plant cultivars (DiGiovanni et al., 1999; Siciliano & Germida, 1999; Dunfield & Germida, 2001). However, this trend is not always observed when examining specific groups of organisms such as the root-associated pseudomonads. Lottman and Berg (2001) used FAME to examine the effects of transgenic and non-transgenic potato cultivars on rhizosphere bacteria. They found that root-associated pseudomonads were homogeneous across cultivars. The same homogeneity was observed for pseudomonads associated with the roots of rice plants (Rangarajan et al., 2001).

Germida et al. (1998a) proposed that canola endophytic bacteria are a result of rhizosphere bacteria moving into the root interior and thus are a subset of the bacterial community found in the rhizosphere. This is confirmed by the results of our phylogenetic analysis. Phylogenetically, the pseudomonad isolates were homogeneous between root zones confirming the suggestion of other authors that they are a subset of the rhizosphere bacteria (Kloepper et al., 1992; Lilley et al., 1996; Germida et al., 1998a). For example, the highest bacterial densities are observed in the roots and decrease progressively from the stem to the leaves (Lamb et al., 1996; Quadt-Hallman and Kloepper, 1996). Independent of the initial inoculum size, endophytic populations tend to approach optimal densities depending on the plant tissue. The fact that colonization is especially abundant in root tissue may reflect the fact that the root is the primary site where endophytes gain entry into plants. This might explain the close relationship between endophytic and rhizosphere colonizing bacteria, that is, many facultative endophytic bacteria can also survive as rhizosphere bacteria.

Examination of the phenotypic traits and the FAME profiles of the same subset of pseudomonads by PCA indicated that minimal variation existed between the isolates from different cultivars (Figure 4.1 & 4.2). For example, no significant variation in the phenotypic properties or the FAME profiles was observed between isolates from the Excel, Innovator and Exceed cultivars,

but slight variation was observed between the phenotypic properties of isolates from the cultivar, Quest, and the remaining cultivars. The variation of *Pseudomonas* isolates from the Quest cultivar was not observed when the FAME profiles were examined. Siciliano and Germida (1999) examined the rhizosphere communities associated with three cultivars of canola, namely Quest, Parkland and Excel. PCA of the FAME profiles from rhizosphere bacteria separated the bacteria isolated from the cultivar Quest from the cultivars Parkland and Excel. Misko and Germida (2002) found that pseudomonads associated with different plant cultivars were metabolically different. This inconsistency could be due to examination of a specific group of rhizosphere bacteria, such as members of the genus *Pseudomonas*, as compared to examination of all members of the rhizosphere microbial community. It is possible that phenotypic variation exists between pseudomonads from different cultivars, but examination of rhizosphere microorganisms at the community level does not detect such differences.

PCA also determined that there was significant variation between the phenotypes of pseudomonads isolated from the root interior and the rhizosphere. This adds to the hypothesis that endophytic bacteria are a subset of rhizosphere bacteria. Despite being phylogenetically homogeneous between root zones, the isolated pseudomonads are phenotypically different when examining the populations from different root zones. Assuming the hypothesis is correct it would be reasonable to expect that the isolates from the root interior and the rhizosphere are phylogenetically homogeneous. The separation in phenotype could explain their ability to colonize the root interior. That is, by phenotypic adaptation, the pseudomonad populations present in the rhizosphere were able to colonize the root-interior. The use of phenotypic variation as a colonization strategy has been observed for *P. brassicacearum* in the colonization of *Brassica napus* roots and thus further supports the hypothesis (Achouak et al., 2004).

As indicated in Table 4.1, the properties most influencing the principal components were the metabolism of select substrates such as melibiose, succinaminic acid, N-acetyl-D-glucosamine, gentiobiose, maltose, β-methyl-D-glucoside, D-raffinose, bromosuccinic acid, L-threonine, glucose-1-phospate and glucose-6-phosphate. Of the above substrates, maltose, D-raffinose, and L-threonine are reported as being components of root exudates of herbaceous plants (Campbell et al., 1997). Many of the substrates that influenced the PCA were substrates not associated with root exudation. Similarly, Misko and Germida (2002) found that the majority of Biolog™ substrates strongly influencing their analysis were substrates not reported as constituents of root exudates. The influence of "non-rhizosphere" carbon substrates on the phenotypic variation observed within the subset of root-associated pseudomonads suggests that the observed variation between isolates from different root zones is not solely due to the influence of root exudation. However, the composition of canola root exudates has not been assessed, so it is not known if the "rhizosphere" substrates used in this study are actually produced by canola roots. In addition, analysis of the data set using ordination analysis could have further elucidated the relationships between the variables examined.

Cluster analysis of the phenotypic properties of the subset of 133 rhizosphere pseudomonads exhibited little correlation with their phylogenetic relationships (Figures 4.9-4.11). In the majority of situations, the isolates grouped into a phylogenetic cluster that had less than 75-80% similarity among their phenotypic traits despite a close evolutionary relationship as determine by partial 16S rRNA and *cpn*60 gene sequencing. For example, pseudomonad isolates 4-101 and 4-13 that are phylogenetically identical exhibit little correlation among their phenotypic properties (~76% phenotypic similarity). This trend is observed for several other pairwise comparisons between isolates such as 5-28 and 5-41, 4-99 and 1-14, and 4-94 and 4-192. The opposite situation whereby pseudomonad isolates that are phylogenetically more distant exhibit high levels of phenotypic

similarity is plausible, but the close phylogenetic relationships of the pseudomonad isolates within the examined population do not permit this observation.

Phenotypic characterization can be considered to represent the end products of evolutionary relationships. Since prokaryotes are asexually reproducing organisms, hypothetically vertical inheritance should predominate, but some bacteria with highly related phylogenetic backgrounds have phenotypic relationships that can no longer be ascertained and vice versa. That is they are polyphyletic meaning that bacteria highly related based on phenotypic characterization can come from different evolutionary backgrounds. Phenotypic incongruence is largely the result of gene loss, gain, modification and lateral gene transfer.

The lateral transfer of the 16S rRNA and the *cpn*60 genes is reported for members of the Archaea (Gogarten et al., 2002), but to the best of my knowledge has not been reported for members of the γ-Proteobacteria. Assuming that the selected genes are acting as 'molecular clocks' for the cell and are rarely transferred (the complexity hypothesis, Jain et al., 1999), there would not be sufficient evolutionary time between some isolates to account for the differences in their phenotypes. Two factors to be considered in the characterization of these isolates are phenotypic drift or variation and erroneous sequence reads. The pooled variance of the phenotypic tests was 7.5% for all 110 unit characters per isolate. The average variance between 16S rRNA gene sequence reads as determined by the re-read of 4 isolates three times was 5% or 27 bases. Accounting for both variances still does not account for the incongruence observed between the phenotype and the chromosomal genotype within the subset of root-associated pseudomonad isolates. Germida et al. (1998b) examined the phenotypic drift of a *P. chlororaphis* strain isolated from the wheat rhizosphere and bulk soil over a growing season. They determined that the influence of drift on the phenotype was minimal. Regardless, all isolates were examined in the same stage of growth and under controlled conditions to minimize variation. The lack of correlation

between phenotype and genotype has not been previously reported among root-associated pseudomonads. A unifying explanation for the gap between phenotype and genotype may be the presence of mutation in operational genes and/or the presence of MGEs within the isolates.

The impact of horizontal gene transfer and thus MGEs was emphasized by Lawrence and Ochman (1998) who found that all phenotypic characteristics distinguishing *E.coli* from *S. enterica* were encoded by horizontally transferred genes. Adaptation through MGEs could be one explanation of the ability of pseudomonads to inhabit different root zones and the observed incongruence between the phylogenetic and the phenotypic positions of the bacteria, but further investigation into the genotype-phenotype dissimilarity is required prior to making any conclusions.

5.0 DIVERSITY AND CHARACTERIZATION OF MGES PRESENT IN ROOT-ASSOCIATED PSEUDOMONADS

5.1 Introduction

Bacteria are prokaryotic organisms that reproduce asexually by cell division. As a result of their reproductive nature, vertical inheritance dominates bacterial evolution with the majority of descendents being clonal with evolutionary changes resulting from rare, incremental changes within the genome. Therefore, phenotypic characters used to classify bacteria should be largely conserved among organisms descended from a common ancestor. However, bacteria such as *Pseudomonas spp.* and *Escherichia coli*, exhibit an extraordinary phenotypic diversity even among species, displaying variable metabolic properties, cellular structures and lifestyles (Souza et al., 1999; Spiers et al., 2000; Martins dos Santos et al., 2004). The ultimate cause of diversification is mutation, but increases in the number of completely sequenced genomes have shown that lateral gene transfer events are common and cannot be dismissed as inconsequential (Doolittle, 1999; Eisen, 2000; Ochman et al., 2000; Koonin et al., 2001; Jain et al., 2003). For example, examination of deviant nucleotide compositions and anomalous phylogenetic distributions in *E.coli* and *Thermotoga maritima* suggest that up to 20% of their genes may have been rapidly acquired by lateral gene transfer (Ochman et al., 2000; Nesbo et al., 2002; Welch et al., 2002). The acquisition of genes by lateral gene transfer is mediated by MGEs such as plasmids, transposons, integrons and bacterophages which can readily lead to taxonomic groups based on phylogenetic criteria being polyphenetic.

MGEs are a major force affecting prokaryotic phenotypic and genetic diversity, but their importance to the adaptation of rhizosphere bacteria is largely unknown. This point was reaffirmed by the previous study (Section 4.0), which showed that the phenotype of root-associated pseudomonads was not reflected by their phylogenetic relatedness. Several studies have reported the occurrence of lateral gene transfer in populations of pseudomonads (Haubold and Rainey, 1996; Kiewitz and Tummler, 2000). Despite lateral gene transfer, a clonal genetic framework remains even from isolates separated spatially and temporally. Nevertheless, the ability of pseudomonads to exploit new environments and to respond to environmental stress is most easily explained by the acquisition of new genes by MGEs. The potential for MGEs to exhibit a large influence on members of the genus, *Pseudomonas*, is deduced from the extensive studies that report the presence of numerous MGEs and atypical DNA sequences within pseudomonads. For example, the presence of large, broad host range plasmids is commonplace among *Pseudomonas spp.* (Sesma et al., 2000; Thomas, 2000). Alfano et al. (2000) reported the presence of tracts of DNA with atypical codon bias indicative of recent gene acquisition in *Pseudomonas syringae*.

This study investigated the distribution and diversity of MGEs associated with a rhizosphere pseudomonad population. Of the 133 pseudomonads, 66 were randomly selected for detailed MGE analysis. Endogenous plasmid isolations, plasmid-mediated isolation of transposons, prophage induction assays and PCR reactions designed to amplify transposable elements and integrons were conducted.

5.2 Materials and Methods

5.2.1 *Pseudomonas* isolates

The *Pseudomonas* isolates assessed were a randomly selected subset (N=66) of the isolates described previously (Section 3.2.1). For the prophage induction assay, *Pseudomonas aeruginosa* PAO1 lysogenic for phage F116 was used as a control strain.

5.2.2 Endogenous plasmid isolations

Endogenous plasmid isolations were conducted using the in-well lysis method described by Pedraza and Diaz-Ricci (2002). Briefly, cells of an overnight culture (O.D. 600nm = 1) were washed twice with cold 0.3% (v/v) sarkosyl in Tris-borate buffer and centrifuged (14,000 rcf, 7 min). The cell pellet was re-suspended in 20 µL of lysis solution (10% sucrose, 10 µL mL^{-1} in 1× Tris-borate EDTA buffer (in g l^{-1}: Tris, 10.8, EDTA, 0.93, boric acid, 5.5, pH 8.0) and 1 µg mL^{-1} of lysozyme) and mixed with 30 µL of 6× loading buffer (1 mL of 2.5% bromophenol blue, 1 mL of 2.5% xylene cyanol, 3 mL of glycerol, 5 mL of water). Samples were run on agarose gels (0.7%) supplemented with sodium dodecyl sulfate (SDS) (1%) for 1 h at 22 V, 2 h at 33 V and 3 h at 56 V. The gels were stained post-electrophoresis for 30 min with ethidium bromide (0.5 µg mL^{-1}) and subsequently destained in distilled water for 50 min prior to visualization under ultraviolet light. The relative size of the plasmids was determined by their migration patterns in comparison to high molecular weight plasmid markers. High molecular weight plasmid markers were obtained from *Shigella flexneri* 24570 (216.4, 157.2, 3.95, and 3.0 kb) and *Escherichia coli* V517 (320, 155, 54, 7.2, 5.6, 5.1, 3.9, 3.0, 2.7, and 2.1 kb). Plasmid contents were analyzed using the G-test with William's correction (Costat v. 6.3.111).

5.2.3 Restriction digest of plasmid DNA

The diversity of plasmid DNA within the pseudomonads was assessed by restriction digestion with the enzymes, *Bam*HI and *Pst*I. For restriction digestion, the procedure to extract plasmid DNA was scaled up to obtain a concentration of DNA conducive to restriction analysis. Plasmid DNA bands were excised from the gel and incubated overnight in sterile distilled deionized water. The plasmid DNA was subsequently concentrated and resuspended in 10 µL of sterile distilled deionized water. Restriction digests were conducted according to standard methods (Maniatis et al., 1982). Briefly, 1 µL of the 10× restriction enzyme buffer and 5-10 units of the appropriate restriction enzyme were added to 10 µL of plasmid DNA extract. The restriction digest was then incubated at 37ºC for 2 h. Following digestion, 3 µL of 6x loading buffer (1 mL of 2.5% bromophenol blue, 1 mL of 2.5% xylene cyanol, 3 mL of glycerol, 5 mL of water) was added to the digest to stop the reaction. Thirteen µL of restricted plasmid DNA were loaded into a lane of a 2% agarose gel containing 0.5 µg mL^{-1} of ethidium bromide and run for ca. 1 h at 100 V. For each gel, a 100 bp DNA ladder (Invitrogen) was used to determine the size of the restricted fragments.

5.2.4 PCR amplification of class II transposons

The presence of class II transposons (Tn3, Tn21, Tn501) in the root-associated pseudomonad population was determined by PCR from whole cell lysates (Dahlberg and Hermansson, 1995). Briefly, one colony from each pure culture was suspended in 100 µL of sterile distilled deionized water. To lyze the cells, suspensions were incubated for 10-12 min in a boiling water bath. Following lysis, the whole cell lysate was centrifuged at 14,000 rcf for 30-45 sec to remove the bulk of the cellular debris. While the suspensions were boiling, the PCR reagents were assembled as follows: 25 µL reactions

were run containing 1× reaction buffer, 2.5 mM $MgCl_2$, 100 μM each deoxynucleoside triphosphate, 0.1 μM each primer, 0.6 U *Taq* polymerase, 2 uL of DNA template, and distilled deionized water.

All PCRs were performed in a Robocycler® Gradient 96 well Temperature Cycler (Stratagene) using thin-walled 0.2 mL reaction tubes. In the thermal cycler, no oil was necessary as the lid and base were heated. For each set of PCR reactions a series of controls was conducted, a negative control containing all the PCR reagents excluding template DNA and a positive control containing one of either pACYC184::Tn21, pBR322::Tn501 or pMB8::Tn3. The primer sets for each transposon (Tn3 primers, p3I: 5'-AACTGATCTTCCTGACCGTC-3', and p3II: 5'-TATGACCGATACGGCAGGTG-3', Tn21 primers, p5II: 5'-TACTGCCGCGCATCAAGATC-3', and p2II: 5'-AGAAAGTTCGTCCTGGGCTG-3', Tn501 primers, p5I: 5'-ACATAGGTGGAATCGCGCAC-3', and p5II: 5'-TACTGCCGCGCATCAAGATC-3') were designed to amplify the internal regions of the *tnpA* genes (Dahlberg and Hermansson, 1995). The PCR cycle used to amplify the internal transposase region (*tnpA*) was: 5 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 1 min at 57°C, 30 sec at 72°C and a final extension time of 10 min at 72°C.

PCR amplification of the *tnpA* gene segments resulted in products of single bands ranging from 411-487 bp. To visualize the PCR products, a 1% agarose gel made in 1× TBE with 0.5 μg ml^{-1} of ethidium bromide was used. Ten μL were taken from each PCR reaction combined with 3 μL of 6× loading buffer and loaded into a well on the gel. For each gel, a 100 bp DNA ladder was used to determine the size of the PCR product. The loaded gels were run in 1× TBE buffer for 1 h at 110 V. The bands were subsequently

visualized by ultraviolet light. Transposon contents were analyzed using the G-test with William's correction (Costat v. 6.3.111).

5.2.5 pGBG1 transposon isolations

As described by Schneider et al. (2000), plasmid pGBG1 was used to exogenously isolate transposable elements within the root-associated pseudomonads. Plasmid pGBG1 was introduced into the pseudomonad isolates by electrotransformation (1.8 kV, 4.5-5 msec). Electrotransformants were selected on Luria Bertani (LB) agar containing 100 μg mL^{-1} of chloramphenicol (Cm) (pGBG1 contains a chloramphenicol resistance gene). The presence of the pGBG1 plasmid within the isolates was confirmed by PCR using the G11, TATCAGCTATGCGCCGACCAGAAC, and G12, GCCAATCCCCATGGCATCGAGTAA, primer set and by plasmid extraction. Because the selection cassette in pGBG1 is based on a tetracycline (Tc) resistance marker, the pseudomonad isolates were checked for intrinsic resistance and the minimum inhibitory concentrations were determined as described by Schneider et al. (2000). Mutation assays were then performed to isolate pGBG1 mutant plasmids. Briefly, electrotransformants were grown overnight in Luria-Bertani (LB) broth containing 100 μg mL^{-1} of Cm. The cultures were then titered on LB + Cm and 0.1 mL of each separate culture was spread onto a LB + Tc (25 μg mL^{-1}) plate. After 48 h of incubation at 28ºC, the mutation frequency was calculated as the number of Tc resistant mutants per bacteria forming colonies. The Tc resistant mutants were further analyzed by plasmid extraction and restriction digestion with *Pst*I as described by Schneider et al. (2000).

5.2.6 PCR amplification of class I integrons

The presence of class I integrons in the selected root-associated pseudomonad population was determined by PCR from whole cell lysates (Lévesque et al., 1995). Briefly, one colony from each pure culture was suspended in 100 μL of sterile distilled deionized water. To lyse the cells, suspensions were incubated for 10-12 min in a boiling water bath. Following lysis, the whole cell lysate was centrifuged at 14,000 rcf for 30-45 sec to remove the bulk of the cellular debris. While the suspensions were boiling, the PCR reagents were assembled as follows: 100 μL reactions were run containing 1× reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 1 mg gelatin per mL), 200 μM of each deoxynucleoside triphosphate, 250 μM of each primer, 3 U of *Taq* polymerase, 30 μL of DNA template, and distilled deionized water.

All PCRs were performed in a Robocycler® Gradient 96 well Temperature Cycler (Stratagene) using thin-walled 0.2 mL reaction tubes. In the thermal cycler, no oil was necessary as the lid and base were heated. For each set of PCR reactions a series of controls was conducted, a negative control containing all the PCR reagents excluding template DNA, and positive controls containing pCER100 (In4), RGN238 (In8), and RIP71a (In2) DNA. Oligonucleotide primers targeted to class I integrons were used to determine their frequency in the isolates and to estimate the size of the inserted DNA. The primers 5'CS, GGCATCCAAGCAGCAAG, and 3'CS, AAGCAGACTTGACCTGA, are complementary to the conserved regions flanking the inserted DNA (Lévesque et al., 1995). The PCR cycle used to amplify the integrons was: 12 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 5 min at 72°C. Five seconds were added to the extension time at each cycle.

To visualize the PCR products, a 0.8% agarose gel made in 1× TBE with 0.5 μg mL^{-1} of ethidium bromide was used. Ten μL were taken from each PCR reaction combined with 3 μL of 6× loading buffer and loaded into a well on the gel. For each gel, a 100 bp DNA ladder was used to determine the size of the PCR product. The loaded gels were run in 1× TBE buffer for 1 h at 110 V. The bands were subsequently visualized by ultraviolet light. Integron contents were analyzed using the G-test with William's correction (Costat v. 6.3.111).

5.2.7 Prophage induction assay

Starter cultures of each isolate were incubated overnight in a shaker-incubator set at 28°C and 150 rpm. One milliliter of each starter culture served as an inoculum for 100 mL of sterile LB broth medium and was incubated at 28°C with shaking at 150 rpm. Growth was monitored every two hours from time 0 to time 10 h using OD_{600nm}. Four hours post-inoculation ($OD_{600nm} \sim 0.4$), the treatment flasks were amended with mitomycin C to a final concentration of 0.5 μg mL^{-1}. The mitomycin C concentration was selected so that a gradual decrease in culture optical density (0.2-0.4 units) occurred within 6 h after addition to the lysogen control strain. Data were analyzed using paired T-tests to compare control versus mitomycin C treated cultures using Minitab v.12 (Minitab Inc., State College, PA). Prophage contents were analyzed using the G-test with William's correction (Costat v. 6.3.111).

5.3 Results

5.3.1 Distribution and diversity of plasmids associated with rhizosphere pseudomonads

Fifty eight percent (N=38) of the 66 *Pseudomonas* isolates screened contained at least one large plasmid (>50 kb) (Table 5.1 & 5.2). An example of a plasmid profile gel is presented in Figure 5.1. Examination of the plasmids contained within the subset of pseudomonads revealed no significant difference in the number of plasmid-containing isolates between root zones (P = 0.625). However, there was a significant difference in the number of plasmid-containing pseudomonads between the cultivars Exceed, Innovator, and Quest, and Excel cultivar (P = 0.001). The majority of plasmid-containing isolates contained a plasmid around 80 kb that was analyzed with the restriction enzymes, *Bam*HI and *Pst*I. Similarity was found between the restriction digests of the 80 kb plasmids showing similar banding patterns for the larger fragments with less similarity observed among the smaller fragments, which suggested that these plasmids are potentially related in their content and in their molecular organization (Figure 5.2).

5.3.2 Distribution and diversity of transposons associated with rhizosphere pseudomonads

Twenty three percent (N=15) of the 66 isolates screened contained a transposon belonging to the Tn3 family (Table 5.1 and 5.2). The number of pseudomonad isolates

Table 5.1. Number of *Pseudomonas* isolates containing a MGE from different cultivars and root zones.

MGE	Number of *Pseudomonas* isolates								No. of isolates	Percent
	Exceed		Excel		Innovator		Quest			
	E[a]	R[b]	E	R	E	R	E	R		
Plasmids	7	7	3	0	6	3	4	8	38	58%
Prophages	3	8	3	2	7	2	6	2	33	50%
Integrons	4	3	0	2	2	2	0	3	16	24%
Transposons[c]	0	4	1	7	2	1	0	0	15	23%

[a]E = root interior
[b]R = rhizosphere
[c]Tn3 family

Table 5.2. Survey of the MGEs associated within 66 pseudomonads isolated from the roots of field-grown canola.

Isolate code	Plasmid content		Integron content		Transposon content			pGBG1 transposon isolations	Prophage induction
	No.	Size (kb)	No.	Size (kb)	Tn3	Tn21	Tn501		
1-6	1	160	-	-	-	-	-	-	-
1-8	1	80	-	-	-	-	-	-	+
1-11	1	80	-	-	-	-	-	-	+
1-12	-	-	1	5.0	-	+	+	+	-
1-14	1	80	-	-	-	-	-	-	+
1-15	1	80	-	-	-	-	-	-	-
1-18	-	-	1	1.0	-	-	-	+	+
2-2	1	80	-	-	-	-	-	-	+
2-6	2	80, 150	-	-	-	-	-	-	-
2-20	1	80	-	-	-	-	-	-	+
2-22	-	-	1	1.0	-	-	-	-	-
4-4	1	80	2	0.7, 2.0	-	-	-	-	+
4-5	1	80	3	0.7, 2.0, 2.5	-	-	-	+	+
4-10	-	-	-	-	-	-	-	-	+
4-11	1	80	-	-	-	-	-	-	+
4-13	-	-	1	1.0	-	+	+	-	-
4-16	-	-	1	0.9	-	-	-	-	-
4-21	1	80	-	-	-	-	-	+	+
4-23	1	80	-	-	-	-	-	+	+
4-32	-	-	1	0.8	-	-	-	-	-
4-36	1	80	-	-	-	+	-	-	+
4-44	1	80	-	-	-	-	-	+	+
4-45	-	-	-	-	-	-	-	+	+
4-51	-	-	-	-	-	-	-	+	+
4-57	-	-	-	-	-	-	-	-	-
4-58	1	80	-	-	-	-	-	+	+
4-62	2	60, 80	-	-	-	-	-	-	-
4-63	2	60, 80	1	2.0	-	-	-	-	-
4-65	1	80	-	-	-	-	-	-	-
4-94	1	80	1	1.0	-	-	-	-	+
4-95	1	60	1	1.5	-	-	-	-	+
4-98	1	60	-	-	-	-	-	-	-
4-99	-	-	-	-	-	+	+	-	+
4-101	-	-	-	-	-	+	-	-	-
4-105	-	-	-	-	-	-	-	-	-
4-107	1	80	-	-	-	-	-	-	-
4-108	-	-	-	-	-	-	-	-	-
4-110	-	-	-	-	-	+	-	-	-
4-114	1	80	-	-	-	-	+	-	-
4-115	-	-	1	1.0	-	-	+	-	+
4-140	1	80	-	-	-	-	-	-	-
4-141	-	-	-	-	-	-	-	-	-
4-143	1	80	1	1.5	-	-	-	-	+

Table 5.2. continued.

Isolate code	Plasmid content		Integron content		Transposon content			pGBG1 transposon isolations	Prophage induction
	No.	Size (kb)	No.	Size (kb)	Tn3	Tn21	Tn501		
4-152	-	-	-	-	-	-	-	+	+
4-180	1	80	-	-	-	-	-	-	+
4-183	1	80	-	-	-	-	-	-	+
4-192	-	-	-	-	-	+	-	-	+
5-2	-	-	-	-	-	-	-	+	+
5-4	1	80	-	-	-	-	-	-	+
5-6	1	80	-	-	-	+	-	-	-
5-7	-	-	-	-	-	+	-	-	+
5-10	-	-	-	-	-	-	-	-	-
5-15	-	-	-	-	-	-	-	-	-
5-18	1	75	-	-	-	-	-	-	-
5-22	2	75, 80	1	0.5	-	-	-	-	-
5-25	1	60	-	-	-	-	-	-	-
5-28	2	60, 80	-	-	-	-	-	+	-
5-30	1	60	1	1.5	-	-	-	-	-
5-40	-	-	-	-	-	+	-	+	-
5-41	-	-	-	-	-	+	-	-	-
5-44	-	-	-	-	-	-	-	-	-
5-45	-	-	-	-	-	-	-	+	-
5-111	1	80	1	1.0	-	-	-	-	+
5-112	-	-	-	-	-	-	+	-	+
5-113	1	80	-	-	-	-	-	-	+
5-117	1	80	-	-	-	+	-	-	+

containing a Tn3-type transposon from the Excel cultivar was significantly higher than isolates from other cultivars ($P<0.05$). In addition, the number of transposons was significantly higher in rhizosphere isoltes as opposed to endophytic isolates ($P<0.05$). The detection of transposable elements within the pseudomonad isolates by the pGBG1 trapping vector indicated that 14 of the 66 isolates (21%) contained at least one transposon. However, the trapping vector detected transposons in isolates that did not show a positive PCR reaction using class II transposon PCR primers and vice versa indicating that the different methods were assessing different portions of the transposon population within the isolates.

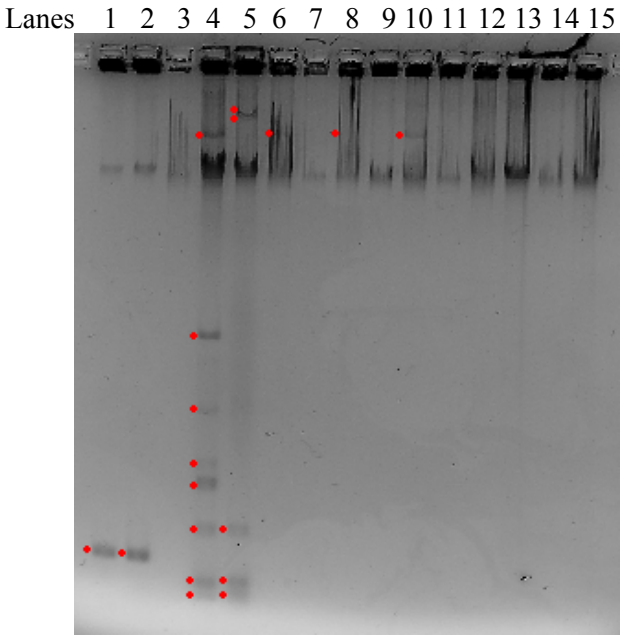

Figure 5.1. Plasmid profiles of pseudomonad isolates from the root interior of the cv. Exceed. The plasmid profiles were separated by electrophorsis in 0.7% agarose supplemented with 1% SDS. Lane 1, pGAD (5-10 kb), lane 2, pGBT (5-10 kb), lane 3, pADPTel (111 kb), lane 4, Escherichia coli V517 (320, 155, 54, 7.2, 5.6, 5.1, 3.9, 3.0, 2.7, and 2.1 kb), lane 5, Shigella flexneri 24570 (216.4, 157.2, 3.95, and 3.0 kb), lanes 6-15, pseudomonad isolates.

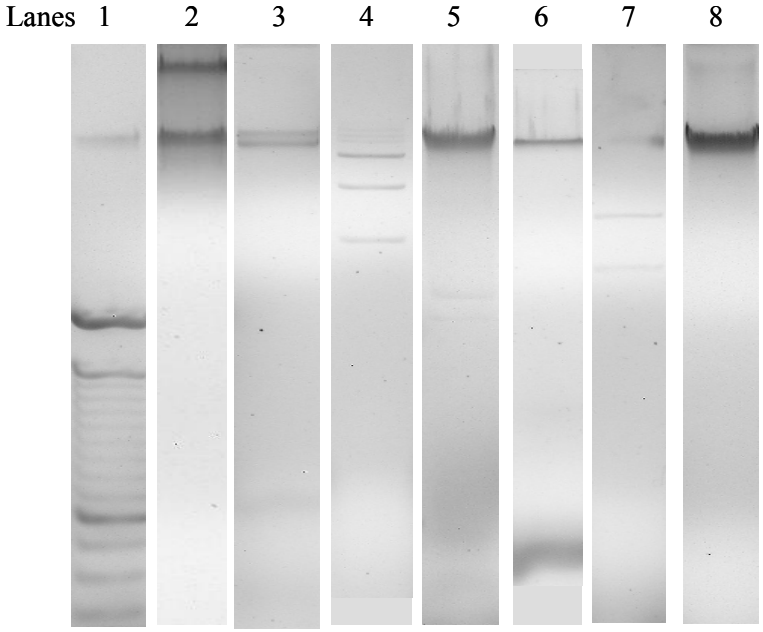

Figure 5.2. Restriction digestion of plasmid DNA contained within pseudomonad isolates. Lane, 1, DNA ladder (60, 2, 1.5, 1.4, 1.3, 1.2, 1.1, 1.0, 0.9, 0.7, 0.6, 0.5, 0.4, and 0.3kb), lanes 2-8, restriction digests of plasmid DNA contained within the pseudomonad isolates.

5.3.3 Distribution and diversity of integrons associated with rhizosphere pseudomonads

Detection of class I integrons by PCR from 66 isolates has indicated that 24% (N=16) contained integrons (Table 5.1 & 5.2). Examination of the data revealed no significant difference in the number of integron-containing isolates between root zones or cultivars.

5.3.4 Prevalence and distribution of inducible prophages associated with rhizosphere pseudomonads

Fifty percent (N=33) of the 66 *Pseudomonas* isolates screened were shown to contain at least one inducible prophage (Table 5.1 & 5.2). The data revealed no significant difference in the number of prophage-containing isolates between root zones or cultivars.

5.3.5 Comparison of the observed phenotypic variation and MGE prevalence within a subset of root-associated pseudomonads

In section 4, the variability within a population of pseudomonads (N=133) isolated from the rhizosphere and root-interior of field-grown canola was assessed by their partial 16S rRNA and *cpn*60 gene sequences and select phenotypic properties (FAME profiles, antibiotic resistance, extracellular enzyme production and carbon substrate utilization). Comparison of the phylogenetic and phenotypic relationships has indicated that the genotype, as reflected by the analyzed gene sequences, was not accurately reflected by their phenotypic relationships. Analysis of the MGE contents and distributions within the pseudomonad population have shown *Pseudomonas* species to contain a large, heterogeneous MGE population varying in frequency and distribution among the isolates

Table 5.3 Examples of MGE contents between *Pseudomonas* isolates with varying degrees of phylogenetic and phenotypic similarity.

Isolates	% 16S rRNA and *cpn*60 Similarity	% Phenotypic Similarity	MGE content			
			Plasmids	Transposons	Integrons	Prophages
4-13	~100	~76	-	Tn21, Tn501	1 (1.0kb)	-
4-101			-	Tn21	-	-
5-28	~100	<60	2 (60, 80kb)	1 (pGBG1 isolation)	-	-
5-41			-	Tn21	-	-
1-14	~100	~76	1 (80 kb)	-	-	+
4-99			-	Tn21, Tn501	-	+
4-94	~100	<60	1 (80 kb)	-	1 (1.0 kb)	+
4-192			-	Tn21	-	+
1-15	~100	<60	1 (80kb)	-	-	-
2-6			2 (80, 150kb)	-	-	-
4-4	~100	~85	1 (80kb)	-	2 (0.7, 2.0kb)	+
4-5			1 (80kb)	-	3 (0.7, 2.0, 2.5kb)	+
4-180	~100	<60	1 (80kb)	-	-	+
4-183			1 (80kb)	-	-	+
1-11	~100	~100	1 (80kb)	-	-	+
4-44			1 (80kb)	1 (pGBG1 isolation)	-	+
4-36	~100	~80	1 (80kb)	Tn21	-	+
5-6			1 (80kb)	Tn21	-	-
4-62	~100	~92	2 (60, 80kb)	-	-	-
4-63			2 (60, 80kb)	-	1 (2.0kb)	-
1-8	~100	<80	1 (80kb)	-	-	+
1-11			1 (80kb)	-	-	+

tested. Comparison of the MGE contents and the observed phenotypic variation suggests that MGEs were present or absent irrespective of the degree of similarity between the phenotypic and phylogenetic relationships and thus could not resolve the variation observed within the isolates (Table 5.3). For example, isolates with high phylogenetic relatedness (~100% gene sequence similarity) and relatively low phenotypic similarity (<75% phenotypic similarity), such as isolates 4-94 and 4-192, contained a variety of different MGEs. Whereas, isolates with high phylogenetic and phenotypic similarities, such as isolate combinations 4-5 and 4-5, and 1-11 and 4-44, also contained a variety of MGEs. A third situation was also observed whereby isolates with high phylogenetic

relatedness and relatively low phenotypic similarity, such as 4-180 and 4-183, exhibit identical MGE profiles.

5.3 Discussion

The evolutionary trajectory and the ability of *Pseudomonas spp*. to adapt to diverse habitats reflects a high functional diversity. Misko and Germida (2002) examined the taxonomic and functional diversity of pseudomonads isolated from canola roots. They found the population to be functionally diverse with members of the same strain exhibiting different metabolic properties. Martins dos Santos et al. (2004) examined the genomic basis for niche specificity in *Pseudomonas putida* KT2440 and found the large functional diversity exhibited by *P. putida* to be reflected in a highly mosaic genome structure (many regions of atypical composition) and in the multiplicity of mobile genetic elements present. In this study, the distribution and diversity of MGEs associated within a subset of rhizosphere pseudomonads was examined. Examination of the prevalence and diversity of MGEs within this subset of root-associated pseudomonads have shown *Pseudomonas* species to be heterogeneous in the frequencies of strains bearing MGEs, with plasmid-bearing strains being 58%, with prophage-bearing strains being 50%, with integron-bearing strains being 24% and with transposon-bearing strains being 23% of the tested strains. In agreement with Martins dos Santos, the genomes of the *Pseudomonas* isolates were found to be very heterogeneous, containing a multiplicity of MGEs.

The MGE most prevalent among the pseudomonad isolates was plasmids, with plasmid-bearing strains being 58% of the isolates tested. Plasmids have been demonstrated to be commonplace among *Pseudomonas spp*., often being very large with broad host ranges (outside the genus) and frequently encoding complete pathways plus

associated regulatory machinery (Sesma et al., 2000; Thomas, 2000; Vivian et al., 2001). In this study, no difference in the number of plasmid-containing isolates was observed between root zones. However, there was a difference in the plasmid content between isolates from the transgenic cultivars (Exceed, Innovator, and Quest), and the non-transgenic cultivar (Excel). To date no reports have focused on the differences in plasmid contents between isolates from transgenic and non-transgenic cultivars and the experimental design of this study was not explicitly designed to test the influence of genetically modified plants on the prevalence of plasmids within root isolates as only one non-transgenic cultivar was examined. In addition, this study was limited to observation of a *Brassica* species and it is possible that different plants (modified and non-modified) may have a different influence on plasmid prevalence.

A 80kb plasmid that was present in a large portion of the isolated pseudomonads was chosen for further restriction analysis. Similarity was found between the restriction digest of the 80kb plasmids showing similar banding patterns for the larger fragments with less similarity observed among the smaller fragments, which suggested that these plasmids might be related in their content and in their molecular organization. The high presence and transfer of plasmids within rhizosphere bacterial populations is thought to facilitate adaptation, but despite their importance, knowledge of the encoded traits is limited due to lack of selectable phenotypes and gene identifications. The high prevalence and relatedness of the isolated plasmids suggests a potential functioning in adaptation to the root environment by these isolates.

The literature suggests that it is not rare for prophages to constitute a sizeable portion of the total bacterial DNA. For example, *P. aeruginosa* PAO1 was reported to

contain two prophages (Stover et al., 2000) and *P. putida* KT2440 was reported to contain four prophages within its genome sequence (Nelson et al., 2002; Canchaya et al., 2003). Seventy one percent (N=40) of the genome sequences examined by Canchaya et al. (2003) contained prophage sequences exceeding 10kb in length. In addition to lysogenic phages contributing to the sizeable prophage contents of some bacterial genomes, defective phage or segments of phage DNA from erroneous excision events will contribute to the total phage DNA contained with a bacterial genome. In our study, 50% of the pseudomonad isolates harbored an inducible prophage irrespective of cultivar or root zone. The acquisition of whole-genome sequences of *Pseudomonas spp*. by other researchers confirms our finding of high prophage prevalence making prophage containing strains the majority.

Amplification of class I integrons from the pseudomonad isolates indicated that 24% of the 66 isolates contained an integron irrespective of cultivar or root zone. The majority of reports focus on integrons as they relate to antimicrobial resistance spread rather than as they relate to the environment, but an interesting report by Micheal et al. (2004) estimated the diversity of integrons in a 5 × 10m soil transect at 2343 types representing a potentially significant evolutionary force.

Of the 66 *Pseudomonas* isolates screened 23% contained a transposon belonging to the Tn3 family (Table 5.1 and 5.2). The number of pseudomonad isolates containing a Tn3-type transposon from the Excel cultivar was significantly higher than isolates from the other cultivars. In addition, the number of transposons was significantly higher in rhizosphere isolates as opposed to endophytic isolates. The detection of transposable elements within the pseudomonad isolates by the pGBG1 trapping vector indicated that

21% contained at least one transposon. However, the trapping vector detected transposons in isolates that did not show a positive PCR reaction using class II transposon PCR primers and vice versa indicating that the different methods were accessing different portions of the MGE population within the isolates. Since the sequence of the trapping vector is known, the transposable elements captured by the plasmid, but not detected by PCR could be sequenced and classified by generating primers to the vector. The presence and ecological importance of transposons, especially catabolic transposons, has been reviewed by Tan (1999). In addition, analysis of the *Pseudomonas putida* KT2440 genome has revealed the presence of multiple insertion sequences and transposons (Martins dos Santos et al., 2004). Nelson et al. (2002) sequenced the complete genome sequence of *Pseudomonas putida* KT2440 and found 41 transposase genes within the genome. Within the 41 transposase gene sequences, 7 encoded novel multicopy insertion sequence (IS) elements. This suggests that in addition to plasmids, prophages, and integrons, transposons are present in high numbers and that methodology used in their detection greatly influences the resulting transposon population detected. The presence of MGEs in 89% of the tested pseudomonad was determined using endogenous plasmid isolations, plasmid-mediated isolation of transposons, transposon PCR, integron PCR, and prophage induction analysis. The limitations of these methodologies, such as primer specificity and the required induction of prophages, could have resulted in MGEs being detected in the remaining 11%

The data shows a high level of diversity among the MGEs of pseudomonads isolated from the roots of field-grown canola characteristic of the genus. The conditions of relatively high selective pressure in the soil and the rhizosphere could provide selective

pressure for *Pseudomonas* species containing MGEs that provide a competitive advantage or provide access to other niches. To date no studies have correlated the presence of MGEs to rhizosphere competence. Further examination of their associated MGEs will aid our understanding of these cryptic elements and the influence they have on the bacterial genome and adaptability.

6.0 SUMMARY AND CONCLUSIONS

In this study the physiological properties and the phylogenetic relationships of a subset of root-associated pseudomonads were examined as well as the diversity of their associated mobile genetic elements (MGEs). On the basis of 16S rRNA and *cpn*60 gene sequences, two major clusters were observed, the *Pseudomonas fluorescens* complex and the *P. putida* complex. Analysis of the pairwise similarities between isolates based on comparison of the *cpn*60 and 16S genes suggested that the evolution rates of the two genes were similar for each isolate and accurately represent the evolution of the isolate. Given their correlation, it would be expected that comparison of each sequence from the same isolate would lead to the same identification, but this was not the case and reaffirms the need for further taxonomic investigation into *Pseudomonas spp*. Furthermore, the resolution between the isolates was slightly greater using the 16S rRNA sequence data as is indicated by a wider range of pairwise similarities as compared to those obtained using the *cpn*60 pairwise similarities. Weak correlation was found between the similarity indexes of the genes and the FAME profiles. The weak congruence could be the result of divergent evolution rates between functional and operational or "essential" genes, the presence of MGEs, inaccuracies in the identification method or the lack of correlation between phenetic and genetic based OTU. The results of our study confirmed the genus identifications of Misko and Germida (2002) and also the need for further taxonomic investigation within the fluorescent pseudomonads.

In accordance with the FAME analysis conducted by Misko and Germida (2002), phylogenetic analysis of the partial 16S rRNA and *cpn*60 gene sequences suggested that the examined subset of pseudomonads found in the different root zones and different canola cultivars was homogeneous. Germida et al. (1998a) proposed that canola endophytic bacteria are a result of rhizosphere bacteria moving into the root interior and thus are a subset of the bacterial community found in the rhizosphere. This was confirmed by the results of our phylogenetic analysis. Principal component analysis (PCA) of their phenotypic properties revealed little variation among the pseudomonads associated with different canola cultivars. Importantly, while little difference was observed between cultivars significant phenotypic variation was observed between the root zones. This adds to the hypothesis that endophytic bacteria are a subset of rhizosphere bacteria as proposed by Germida et al. (1998a). Despite being phylogenetically homogeneous between root zones, the isolated pseudomonads were phenotypically different when the populations from different root zones were examined. Assuming the hypothesis is correct it would be reasonable to expect that the isolates from the root interior and the rhizosphere would be phylogenetically homogeneous. The separation in phenotype could explain their ability to colonize the root interior. That is, by phenotypic adaptation, the pseudomonad populations present in the rhizosphere were able to colonize the root-interior.

Cluster analysis of their phenotypic properties exhibited little correlation with their phylogenetic relationships. In the majority of situations, the isolates grouped into a phylogenetic cluster had less than ca. 75-80% similarity among their phenotypic traits despite a close evolutionary relationship as determined by 16S rRNA and *cpn*60 gene

sequencing. The results indicated that the genotype of the rhizosphere pseudomonads was not accurately reflected by their phenotype. The lack of correlation between phenotype and genotype has not been previously reported among root-associated pseudomonads. A unifying explanation for the gap between phenotype and genotype may be the presence of mutation in operational genes and/or the presence of MGEs within the isolates.

Examination of the MGEs associated with a randomly selected subset of the pseudomonad isolates (N=66) revealed that 58% (N=38) contained plasmids, 50% (N=33) contained inducible prophages, 24% (N=16) contained integrons and 23% (N=15) contained transposons. MGEs were present in the pseudomonad isolates independent of the root zone or the degree of similarity between the phenotypic and the phylogenetic relationships of the root-associated pseudomonad population. The data shows a high level of diversity among the MGEs of pseudomonads isolated from the roots of field-grown canola characteristic of the genus. The conditions of relatively high selective pressure in the soil and the rhizosphere could provide selective pressure for *Pseudomonas* species containing MGEs that provide a competitive advantage or provide access to other niches. To date no studies have correlated the presence of MGEs to rhizosphere competence.

Overall, a large functional diversity was observed among this subset of root-associated pseudomonads despite very close phylogenetic relationships. The presence of MGEs within isolates that were both close in their phylogenetic and phenotypic positions as well as isolates that were closely related phylogenetically but phenotypically distant indicates that the MGEs do not appear to be the major source influencing the observed

variation between the phylogenetic and the phenotypic relationships of the bacteria examined. Therefore, MGEs do not appear to be responsible for the localization of isolates to a particular root zone or for the discrepancy between the phylogenetic and the phenotypic relationships of the bacteria examined suggesting mutation and genomic rearrangement to be the primary influences on the observed incongruence.

Future experimentation is required to explain the large functional diversity observed among the root-associated pseudomonads and its source. Their large presence in the rhizosphere suggests that they are ecologically significant. Further examination of their functional diversity will aid in the elucidation of the role of pseudomonads in the rhizosphere.

The majority of MGEs examined in our study were not localized to a particular root zone suggesting that MGEs were not solely responsible for the localization of the isolates to a particular root zone or cultivar. Future experimentation into the influence of MGEs on rhizosphere competence would explain the presence and role of these elements in rhizosphere bacteria.

The lack of correlation between the presence of MGEs and the observed phenotypic variation reaffirms mutation and genomic rearrangments as the major evolutionary forces. This study has evolutionary implications in that it begins to explain why, despite the large prevalence and transfer of MGEs among *Pseudomonas spp*., a clonal framework prevails with distinct genetic groupings.

7.0 LITERATURE CITED

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Appendix A: Bacterial isolate codes

Table A.1. Bacterial isolate codes.

Isolate code[a]	Isolate code (Misko, 2002)[b]
1-1	MF E E 3-16
1-3	MF XD E 2-66
1-4	MF XD E 2-9
1-6	MF XD E 1-20
1-7	MF Q E 1-39
1-8	MF Q E 4-53
1-10	MF E E 2-122
1-11	MF I E 1-7
1-12	MF E R 1-66
1-14	MF XD E 1-14
1-15	MF XD E 2-118
1-16	MF I E 1-75
1-17	MF XD E 2-104
1-18	MF I E 2-33
2-1	MF XD E 4-90
2-2	MF E E 1-3
2-3	MF E E 3-9
2-4	MF XD E 3-26
2-5	MF XD E 2-46
2-6	MF XD E 1-9
2-8	MF Q E 4-86
2-9	MF Q E 4-37
2-12	MF XD R 1-121
2-14	MF I E 3-26
2-15	MF XD E 2-79
2-16	MF XD E 2-75
2-19	MF Q E 4-7
2-20	MF I E 4-45
2-22	MF XD E 3-26
4-4	MFXDE2-118a1
4-5	MFXDE2-118a2
4-6	MFXDE2-118a3
4-9	MFEE2-122c1
4-10	MFEE2-122c2
4-11	MFEE2-122c3

[a]Arbitrary isolate code.

[b]Isolate code refers to the plants from which the isolates came. MF indicates the 1998 Melfort field site. The second letter (s) indicates the plant cultivar (E=Excel, I=Innovator, XD=Exceed, Q=Quest). The third letter indicates the root zone from which the isolate was isolated (E=root interior, R=rhizosphere). The first number indicates which of 4 plant replicates the isolate came from. The second number is a randomly assigned number.

Table A.1. continued.

Isolate code	Isolate code (Misko, 2002)
4-12	MFEE2-122c4
4-13	MFER1-66d1
4-15	MFXDE2-9e1
4-16	MFXDE2-9e2
4-19	MFXDE1-20g1
4-20	MFXDE1-20g2
4-21	MFQE4-53i1
4-23	MFQE1-39h1
4-30	MFIE1-120r2
4-31	MFIE1-120r1
4-32	MFIE2-33q2
4-33	MFIE2-33q1
4-36	MF I E 1-75o2
4-44	MFIE1-7k2
4-45	MFQE1-28j2
4-46	MFQE2-40
4-48	MFQE2-71
4-51	MFQE2-65
4-54	MFXDE4-38
4-57	MFQR2-98
4-58	MFQR2-84
4-61	MFQR1-208
4-62	MFQR2-4
4-63	MFQR4-20
4-64	MFQE2-3
4-65	MFQE2-49
4-94	MFIR1-12
4-95	MFQR4-6
4-96	MFQR4-29
4-98	MFQR4-141
4-99	MFER1-4
4-100	MFER1-24
4-101	MFER1-34
4-102	MFER1-101
4-103	MFEE2-35
4-105	MFEE2-4
4-106	MFEE3-24
4-107	MFEE4-27
4-108	MFEE4-78
4-109	MFEE4-71
4-110	MFEE4-40
4-114	MFXDR3-28

Table A.1. continued.

Isolate code	Isolate code (Misko, 2002)
4-115	MFXDR3-80
4-139	MFXDE2-52
4-140	MFXDE2-1
4-141	MFIE4-41
4-143	MFXDR1-25
4-151	MFQE1-5
4-152	MFQE1-2
4-154	MFQE4-12
4-156	MFIE4-87
4-157	MFIE4-35
4-180	MFXDR1-34
4-183	MFXDR1-36
4-184	MFER4-3
4-185	MFER4-112
4-186	MFER3-28
4-192	MFER3-75
4-193	MFXDR1-170
5-1	MFIE4-73
5-2	MFIE3-103
5-3	MFIE3-31
5-4	MFIE2-109
5-5	MFIE3-11
5-6	MFIE1-91
5-7	MFIR1-164
5-9	MFIR1-94
5-10	MFIR2-17
5-15	MFIR3-89
5-17	MFIR4-53
5-18	MFIR4-76
5-19	MFIR4-99
5-22	MFIR4-74
5-25	MFQR4-44
5-27	MFQR3-27
5-28	MFQR4-119
5-30	MFQR3-5
5-31	MFQR1-9
5-36	MFEE2-108
5-37	MFEE4-19
5-39	MFER1-96
5-40	MFER1-29
5-41	MFER1-88
5-42	MFER4-111

Table A.1. continued.

Isolate code	Isolate code (Misko, 2002)
5-44	MFER2-40
5-45	MFER3-43
5-46	MFER4-79
5-108	MFXDR1-122
5-109	MFXDR1-36
5-110	MFXDR1-153
5-111	MFXDR1-59
5-112	MFXDR2-79
5-113	MFXDR2-45
5-115	MFXDR3-121
5-117	MFXDR3-60
5-118	MFXDR3-92
5-119	MFXDR4-12
5-120	MFXDR4-47